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(54) Title: **METHODS AND COMPOSITIONS FOR THE REPAIR AND/OR REGENERATION OF DAMAGED MYOCARDIUM**

(57) Abstract: Methods, compositions, and kits for repairing damaged myocardium and/or myocardial cells including the administration of stem cells, such as adult stem cells, optionally with cytokines are disclosed and claimed.

TITLE OF THE INVENTION***METHODS AND COMPOSITIONS FOR THE REPAIR AND/OR
REGENERATION OF DAMAGED MYOCARDIUM*****RELATED APPLICATIONS/PATENT & INCORPORATION BY****5 REFERENCE**

This application claims priority from Provisional U.S. Patent Applications Serial Numbers 60/221,902 filed July 31, 2000, 60/258,564 filed December 29, 2000 and 60/258,805 filed January 2, 2001, and 60/295,807, 60/295,806, 60/295, 805, 60/295,804, and 60/295,803 filed June 5, 2001.

10 Each of the applications and patents cited in this text, including each of the foregoing cited applications, as well as each document or reference cited in each of the applications and patents (including during the prosecution of each issued patent; "application cited documents"), and each of the PCT and foreign applications or patents corresponding to and/or claiming priority from any of these applications and
15 patents, and each of the documents cited or referenced in each of the application cited documents, are hereby expressly incorporated herein by reference. More generally, various documents or references are cited in this text, either in a Reference List before the claims or in the text itself; and, each of the documents or references ("herein cited documents") and all of the documents cited in this text
20 (also "herein cited documents"), as well as each document or reference cited in each of the herein cited documents (including any manufacturer's specifications, instructions, etc. for products mentioned herein and in any document incorporated herein by reference), is hereby expressly incorporated herein by reference. There is no admission that any of the various documents cited in this text are prior art as to
25 the present invention. Any document having as an author or inventor person or persons named as an inventor herein is a document that is not by another as to the inventive entity herein. Also, teachings of herein cited documents and documents cited in herein cited documents and more generally in all documents incorporated herein by reference can be employed in the practice and utilities of the present
30 invention.

FIELD OF THE INVENTION

The present invention relates generally to the field of cardiology, and more particularly relates to methods and cellular compositions for treatment of a patient suffering from a cardiovascular disease, including, but not limited to, atherosclerosis, ischemia, hypertension, restenosis, angina pectoris, rheumatic heart disease, congenital cardiovascular defects and arterial inflammation and other disease of the arteries, arterioles and capillaries.

Moreover, the present invention relates to any one or more of:

Methods and/or pharmaceutical composition comprising a therapeutically effective amount of somatic stem cells alone or in combination with a cytokine such as a cytokine selected from the group consisting of stem cell factor (SCF), granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), stromal cell-derived factor-1, steel factor, vascular endothelial growth factor, macrophage colony stimulating factor, granulocyte-macrophage stimulating factor or Interleukin-3 or any cytokine capable of the stimulating and/or mobilizing stem cells. Cytokines may be administered alone or in combination of with any other cytokine capable of: the stimulation and/or mobilization of stem cells; the maintenance of early and late hematopoiesis (see below); the activation of monocytes (see below), macrophage/monocyte proliferation; differentiation, motility and survival (see below) and a pharmaceutically acceptable carrier, diluent or excipient (including combinations thereof). The stem cells are advantageously adult stem cells, such as hematopoietic or cardiac stem cells or a combination thereof or a combination of cardiac stem cells and any other type of stem cells.

The implanting, depositing, administering or causing of implanting or depositing or administering of stem cells, such as adult stem cells, for instance hematopoietic or cardiac stem cells or a combination thereof or any combination of cardiac stem cells (e.g., adult cardiac stem cells) and stem cells of another type of (e.g., adult stem cells of another type), alone or with a cytokine such as a cytokine selected from the group consisting of stem cell factor (SCF), granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), stromal cell-derived factor-1, steel factor, vascular endothelial growth

factor, macrophage colony stimulating factor, granulocyte-macrophage stimulating factor or Interleukin-3 or any cytokine capable of the stimulating and/or mobilizing stem cells (wherein "with a cytokine ..." can include sequential implanting, depositing administering or causing of implanting or depositing or administering of the stem cells and the cytokine or the co-implanting co-depositing or co-administering or causing of co-implanting or co-depositing or co-administering or the simultaneous implanting, depositing administering or causing of implanting or depositing or administering of the stem cells and the cytokine), in circulatory tissue or muscle tissue or circulatory muscle tissue, e.g., cardiac tissue, such as the heart or blood vessels – e.g., veins, arteries, that go to or come from the heart such as veins and arteries directly connected or attached or flowing into the heart, for instance the aorta. This implanting, depositing, or administering or causing of implanting, depositing or administering can be in conjunction with grafts. Such implanting, depositing or administering or causing of implanting, depositing or administering is advantageously employed in the treatment or therapy or prevention of cardiac conditions, such as to treat areas of weakness or scarring in the heart or prevent the occurrence or further occurrence of such areas or to treat conditions which cause or irritate such areas, for instance myocardial infarction or ischemia or other e.g., genetic, conditions that impart weakness or scarring to the heart (*see also* cardiac conditions mentioned *infra*).

The use of such stem cells alone or in combination with said cytokine(s), in the formulation of medicaments for such treatment, therapy or prevention.

Medicaments for use in such treatment, therapy or prevention comprising the stem cells and optionally the cytokine(s).

Kits comprising the stem cells and optionally the cytokine(s) for formulations for use in such treatment, therapy or prevention.

Compositions comprising such stem cells and optionally at least one cytokine and kits for preparing such compositions.

Methods of making the kits and compositions described herein.

Methods of implanting or depositing stem cells or causing the implanting or depositing of stem cells.

BACKGROUND OF THE INVENTION

Cardiovascular disease is a major health risk throughout the industrialized world. Atherosclerosis, the most prevalent of cardiovascular diseases, is the principal cause of heart attack, stroke, and gangrene of the extremities, and thereby the principal cause of death in the United States. Atherosclerosis is a complex disease involving many cell types and molecular factors (for a detailed review, see Ross, 1993, Nature 362: 801-809).

Ischemia is a condition characterized by a lack of oxygen supply in tissues of organs due to inadequate perfusion. Such inadequate perfusion can have number of natural causes, including atherosclerotic or restenotic lesions, anemia, or stroke, to name a few. Many medical interventions, such as the interruption of the flow of blood during bypass surgery, for example, also lead to ischemia. In addition to sometimes being caused by diseased cardiovascular tissue, ischemia may sometimes affect cardiovascular tissue, such as in ischemic heart disease. Ischemia may occur in any organ, however, that is suffering a lack of oxygen supply.

The most common cause of ischemia in the heart is myocardial infarction (MI), commonly known as a heart attack, is one of the most well-known types of cardiovascular disease. 1998 estimates show 7.3 million people in the United States suffer from MI, with over one million experiencing an MI in a given year (American Heart Association, 2000). Of these individuals, 25% of men, and 38% of females will die within a year of their first recognized MI (American Heart Association, 2000). MI is caused by a sudden and sustained lack of blood flow to an area of the heart, commonly caused by narrowing of a coronary artery. Without adequate blood supply, the tissue becomes ischemic, leading to the death of myocytes and vascular structures. This area of necrotic tissue is referred to as the infarct site, and will eventually become scar tissue.

Current treatments for MI focus on reperfusion therapy, which attempts to start the flow of blood to the affected area to prevent the further loss of tissue. The main choices for reperfusion therapy include the use of anti-thrombolytic agents, or performing balloon angioplasty, or a coronary artery bypass graft. Anti-thrombolytic agents solubilize blood clots that may be blocking the artery, while balloon angioplasty threads a catheter into the artery to the site of the occlusion, where the tip of the catheter is inflated, pushing open the artery. Still more invasive

procedures include the bypass, where surgeons remove a section of a vein from the patient, and use it to create a new artery in the heart, which bypasses the blockage, and continues the supply of blood to the affected area. In 1998, there were an estimated 553,000 coronary artery bypass graft surgeries and 539,000 percutaneous transluminal coronary angioplastys. These procedures average \$27,091 and \$8,982 per patient, respectively (American Heart Association, 2000).

These treatments may succeed in reestablishing the blood supply, however tissue damage that occurred before the reperfusion treatment began has been thought to be irreversible. For this reason, eligible MI patients are started on reperfusion therapy as soon as possible to limit the area of the infarct.

As such, most studies on MI have also focused on reducing infarct size. There have been a few attempts to regenerate the necrotic tissue by transplanting cardiomyocytes or skeletal myoblasts (Leor et al., 1996; Murray, et al., 1996; Taylor, et al., 1998; Tomita et al., 1999; Menasche et al., 2000). While the cells may survive after transplantation, they fail to reconstitute healthy myocardium and coronary vessels that are both functionally and structurally sound.

All of the cells in the normal adult originate as precursor cells which reside in various sections of the body. These cells, in turn, derive from very immature cells, called progenitors, which are assayed by their development into contiguous colonies of cells in 1-3 week cultures in semisolid media such as methylcellulose or agar. Progenitor cells themselves derive from a class of progenitor cells called stem cells. Stem cells have the capacity, upon division, for both self-renewal and differentiation into progenitors. Thus, dividing stem cells generate both additional primitive stem cells and somewhat more differentiated progenitor cells. In addition to the well-known role of stem cells in the development of blood cells, stem cells also give rise to cells found in other tissues, including but not limited to the liver, brain, and heart.

Stem cells have the ability to divide indefinitely, and to specialize into specific types of cells. Totipotent stem cells, which exist after an egg is fertilized and begins dividing, have total potential, and are able to become any type of cell. Once the cells have reached the blastula stage, the potential of the cells has lessened, with the cells still able to develop into any cell within the body, however they are unable to develop into the support tissues needed for development of an embryo.

The cells are considered pluripotent, as they may still develop into many types of cells. During development, these cells become more specialized, committing to give rise to cells with a specific function. These cells, considered multipotent, are found in human adults and referred to as adult stem cells. It is well known that stem cells
5 are located in the bone marrow, and that there is a small amount of peripheral blood stem cells that circulate throughout the blood stream (National Institutes of Health, 2000).

Due to the regenerative properties of stem cells, they have been considered an untapped resource for potential engineering of tissues and organs. It would be an
10 advance to provide uses of stem cells with respect to addressing cardiac conditions.

Mention is made of :

U.S. Patent No. 6,117,675 which relates to the differentiation of retinal stem cells into retinal cells *in vivo* or *in vitro*, which can be used as a therapy to restore vision.

15 U.S. Patent No. 6,001,934 involving the development of functional islets from islets of Langerhans stem cells.

U.S. Patents Nos. 5,906,934 and 6,174,333 pertaining to the use of mesenchymal stem cells for cartilage repair, and the use of mesenchymal stem cells for regeneration of ligaments; for instance, wherein the stem cells are embedded in a
20 gel matrix, which is contracted and then implanted to replace the desired soft tissue.

U.S. Patent Nos. 6,099,832, and 6,110,459 involving grafts with cell transplantation.

PCT Application Nos. PCT/US00/08353 (WO 00/57922) and PCT/US99/17326 (WO 00/06701) involving intramyocardial injection of autologous
25 bone marrow and mesenchymal stem cells which fails to teach or suggest administering, implanting, depositing or the use of hematopoietic stem cells as in the present invention, especially as hematopoietic stem cells as in the present invention are advantageously isolated and/or purified adult hematopoietic stem cells.

Furthermore, at least certain of these patent documents fail to teach or
30 suggest the present invention for additional reasons. The source of the stem cells of interest is limited to the known precursors of the type of tissue for which regeneration is required. Obtaining and purifying these specific cells can be extremely difficult, as there are often very few stem cells in a given tissue. In

contrast, a benefit of the present invention results from the ability of various lineages of stem cells to home to the myocardium damage and differentiate into the appropriate cell types- an approach that does not require that the stem cells are recovered directly from myocardium, and, a variety of types of stem cells may be used without compromising the functionality of the regenerated tissue. And, other of these patent documents utilize stem cells as the source of various chemical compositions, without utilizing their proliferative capabilities, and thereby fail to teach or suggest the invention.

Only recent literature has started to investigate the potentials for stem cells to aid in the repair of tissues other than that of known specialization. This plasticity of stem cells, the ability to cross the border of germ layers, is a concept only in its infancy (Kempermann et al, 2000, Temple, 2001). Kocher et al (2001) discusses the use of adult bone marrow to induce neovascularization after infarction as an alternative therapy for left ventricle remodeling (reviewed in Rosenthal and Tsao, 2001). Other studies have focused on coaxing specific types of stem cells to differentiate into myocardial cells, i.e. liver stem cells as shown in Malour et al (2001). Still other work focuses on the possibilities of bone-marrow derived stem cells (Krause, et al., 2001).

One of the oldest uses of stem cells in medicine is for the treatment of cancer. In these treatments, bone marrow is transplanted into a patient whose own marrow has been destroyed by radiation, allowing the stem cells in the transplanted bone marrow to produce new, healthy, white blood cells.

In these treatments, the stem cells are transplanted into their normal environment, where they continue to function as normal. Until recently, it was thought that any particular stem cell line was only capable of producing three or four types of cells, and as such, they were only utilized in treatments where the stem cell was required to become one of the types of cells for which their ability was already proven. Researchers are beginning to explore other options for treatments of myriad disorders, where the role of the stem cell is not well defined. Examples of such work will be presented in support of the present invention.

Organ transplantation has been widely used to replace diseased, nonfunctional tissue. More recently, cellular transplantation to augment deficiencies in host tissue function has emerged as a potential therapeutic paradigm. One

example of this approach is the well publicized use of fetal tissue in individuals with Parkinsonism (reviewed in Thompson, 1992), where dopamine secretion from transplanted cells alleviates the deficiency in patients. In other studies, transplanted myoblasts from unaffected siblings fused with endogenous myotubes in Duchenne's patients; importantly the grafted myotubes expressed wild-type dystrophin (Gussoni et al., 1992).

Despite their relevance in other areas, these earlier studies do not describe any cellular transplantation technology that can be successfully applied to the heart, where the ability to replace damaged myocardium would have obvious clinical relevance. Additionally, the use of intra-cardiac grafts to target the long-term expression of angiogenic factors and ionotropic peptides would be of therapeutic value for individuals with myocardial ischemia or congestive heart failure, respectively.

In light of this background there is a need for the improvement of myocardial regeneration technology in the heart. Desirably, such technology would not only result in tissue regeneration in the heart but also enable the delivery of useful compositions directly to the heart. The present invention addresses these needs.

It is therefore believed that heretofore the administration, implanting, depositing, causing to be deposited, implanted or administered of stem cells, alone or in combination with at least one cytokine, as well as the use of such stem cells alone or in combination with said cytokine(s), in the formulation of medicaments for treatment, therapy or prevention, as in this disclosure and as in the present invention, has not been taught, or suggested in the art and that herein methods, compositions, kits and uses are novel, nonobvious and inventive, i.e., that the present invention has not been taught or suggested in the art and that the present invention is novel, nonobvious and inventive.

OBJECT AND SUMMARY OF THE INVENTION

It has surprisingly been found that the implantation of somatic stem cells into the myocardium surrounding an infarct following a myocardial infarction, migrate into the damaged area, where they differentiate into myocytes, endothelial cells and smooth muscle cells and then proliferate and form structures including myocardium, coronary arteries, arterioles, and capillaries, restoring the structural and functional integrity of the infarct.

It has also surprisingly been found that following a myocardial infarction, the administration of a cytokine to the patient, stimulates the patient's own stem cells, causing them to enter the blood stream and home to the infarcted area. It has also been found that once the cells home to the infarct, they migrate into the damaged
5 tissue, where they differentiate into myocytes, endothelial cells and smooth muscle cells and then proliferate and form structures including myocardium, coronary arteries, arterioles and capillaries, restoring structural and functional integrity to the infarcted area.

The invention provides to methods and/or compositions for repairing and/or
10 regenerating recently damaged myocardium and/or myocardial cells comprising the administration of somatic stem cells, e.g., adult stem cells or cardiac stem cells or hematopoietic stem cells or a combination thereof, such as adult cardiac or adult hematopoietic stem cells or a combination thereof or a combination of cardiac stem cells and a stem cell of another type, such as a combination of adult cardiac stem
15 cells and adult stem cells of another type.

The invention further provides a method and/or compositions for repairing and/or regenerating recently damaged myocardium and/or myocardial cells comprising the administration of at least one cytokine.

The invention still further relates to a method and/or compositions for
20 repairing and/or regenerating recently damaged myocardium comprising the administration of somatic stem cells, e.g., adult stem cells or cardiac stem cells or hematopoietic stem cells or a combination thereof, such as adult cardiac or adult hematopoietic stem cells or a combination thereof or a combination of cardiac stem cells and a stem cell of another type, such as a combination of adult cardiac stem
25 cells and adult stem cells of another type and a cytokine.

The invention yet further provides a method for preparing any of the aforementioned or herein disclosed compositions comprising admixing the pharmaceutically acceptable carrier and the somatic stem cells and/or cytokines.

The invention also provides to a kit comprising a pharmaceutical
30 composition for use in repairing and/or regenerating recently damaged myocardium and/or myocardial cells.

The invention provides methods involving implanting, depositing, administering or causing the implanting or depositing or administering of stem cells,

such as adult stem cells, for instance hematopoietic or cardiac stem cells or a combination thereof or any combination of cardiac stem cells (e.g., adult cardiac stem cells) and stem cells of another type of (e.g., adult stem cells of another type), alone or with a cytokine such as a cytokine selected from the group consisting of stem cell factor (SCF), granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), stromal cell-derived factor-1, steel factor, vascular endothelial growth factor, macrophage colony stimulating factor, granulocyte-macrophage stimulating factor or Interleukin-3 or any cytokine capable of the stimulating and/or mobilizing stem cells (wherein "with a cytokine ...") can include sequential implanting, depositing administering or causing of implanting or depositing or administering of the stem cells and the cytokine or the co-implanting co-depositing or co-administering or causing of co-implanting or co-depositing or co-administering or the simultaneous implanting, depositing administering or causing of implanting or depositing or administering of the stem cells and the cytokine), in circulatory tissue or muscle tissue or circulatory muscle tissue, e.g., cardiac tissue, such as the heart or blood vessels – e.g., veins, arteries, that go to or come from the heart such as veins and arteries directly connected or attached or flowing into the heart, for instance the aorta. This implanting, depositing, or administering or causing of implanting, depositing or administering can be in conjunction with grafts.

Such implanting, depositing or administering or causing of implanting, depositing or administering is advantageously employed in the treatment or therapy or prevention of cardiac conditions, such as to treat areas of weakness or scarring in the heart or prevent the occurrence or further occurrence of such areas or to treat conditions which cause or irritate such areas, for instance myocardial infarction or ischemia or other e.g., genetic, conditions that impart weakness or scarring to the heart (*see also* cardiac conditions mentioned *supra*).

The invention additionally provides the use of such stem cells alone or in combination with said cytokine(s), in the formulation of medicaments for such treatment, therapy or prevention.

And thus, the invention also provides medicaments for use in such treatment, therapy or prevention comprising the stem cells and optionally the cytokine(s).

Likewise the invention provides kits comprising the stem cells and optionally the cytokine(s) for formulations for use in such treatment, therapy or prevention. The stem cells and the cytokine(s) can be in separate containers in a package or in one container in a package; and, the kit can optionally include a device for
5 administration (e.g., syringe) and/or instructions for administration and/or admixture.

The invention also provides compositions comprising such stem cells and optionally the cytokine(s) and kits for preparing such compositions (e.g., kits comprising the stem cells and optionally the cytokine(s); stem cells and the
10 cytokine(s) can be in separate containers in a package or in one container in a package; and, the kit can optionally include a device for administration (e.g., syringe) and/or instructions for administration and/or admixture), as well as methods of making the aforementioned compositions.

The invention also provides a means of generating and/or regenerating
15 myocardium *ex vivo*, wherein somatic stem cells and heart tissue are cultured in vitro, optionally in the presence of a cytokine. The somatic stem cells differentiate into myocytes, smooth muscle cells and endothelial cells, and proliferate in vitro, forming myocardial tissue and/or cells. These tissues and cells may assemble into cardiac structures including arteries, arterioles, capillaries, and myocardium. The
20 tissue and/or cells formed *in vitro* may then be implanted into a patient, e.g. via a graft, to restore structural and functional integrity.

In this disclosure, “comprises,” “comprising,” “containing” and “having” and the like can have the meaning ascribed to them in U.S. Patent law and can mean “includes,” “including,” and the like; “consisting essentially of” or “consists
25 essentially” likewise has the meaning ascribed in U.S. Patent law and the term is open-ended, allowing for the presence of more than that which is recited so long as basic or novel characteristics of that which is recited is not changed by the presence of more than that which is recited, but excludes prior art embodiments.

These and other embodiments are disclosed or are obvious from and
30 encompassed by, the following Detailed Description.

BRIEF DESCRIPTION OF FIGURES

The following Detailed Description, given to describe the invention by way of example, but not intended to limit the invention to specific embodiments described, may be understood in conjunction with the accompanying Figures, incorporated herein by reference, in which:

Figure 1 shows a log-log plot showing Lin⁻ bone marrow cells from EGFP transgenic mice sorted by FACS based on *c-kit* expression (The fraction of *c-kit*^{POS} cells (upper gate) was 6.4%. *c-kit*^{NEG} cells are shown in the lower gate. *c-kit*^{POS} cells were 1-2 logs brighter than *c-kit*^{NEG} cells)

Figure 2A shows a photograph of a tissue section from a MI induced mouse (The photograph shows the area of myocardial infarct (MI) injected with Lin⁻*c-kit*^{POS} cells from bone marrow (arrows), the remaining viable myocardium (VM), and the regenerating myocardium (arrowheads). Magnification is 12X);

Figure 2B shows a photograph of the same tissue section of Figure 2A at a higher magnification, centering on the area of the MI with magnification being 50X;

Figures 2C, D show photographs of a tissue section at low and high magnifications of the area of MI, injected with Lin⁻*c-kit*^{POS} cells, with the magnification of 2C being 25X, and the magnification of 2D being 50 X;

Figure 2E shows a photograph of a tissue section of the area of MI injected with Lin⁻*c-kit*^{NEG} cells wherein only healing is apparent and the magnification is 50X (*Necrotic myocytes. Red= cardiac myosin; green= PI labeling of nuclei);

Figures 3A-C show photographs of a section of tissue from a MI induced mouse, showing the area of MI injected with Lin⁻*c-kit*^{POS} cells (Visible is a section of regenerating myocardium from endocardium (EN) to epicardium (EP). All photographs are labeled to show the presence of infarcted tissue in the subendocardium (IT) and spared myocytes in the subendocardium (SM). **Figure 3A** is stained to show the presence of EGFP (green). Magnification is 250X. **Figure 3B** is stained to show the presence of cardiac myosin (red). Magnification is 250X.

Figure 3C is stained to show the presence of both EGFP and myosin (red-green), as well as PI-stained nuclei (blue). Magnification is 250X);

Figure 4A shows of grafts depicting the effects of myocardial infarction on left ventricular end-diastolic pressure (LVEDP), developed pressure (LVDP), LV + rate of pressure rise (dP/dt), and LV - rate of pressure decay (dP/dt) (From left to right, bars indicate: sham-operated mice (SO, n=11); mice non-injected with Lin⁻ c-kit^{POS} cells (MI, n=5 injected with Lin⁻ c-kit^{NEG} cells; n=6 non-injected); mice
 5 injected with Lin⁻ c-kit^{POS} cells (MI+BM, n=9). Error bars are the standard deviation. * † p<0.05 vs SO and MI);

Figure 4B shows a drawing of a proposed scheme for Lin⁻ c-kit^{POS} cell differentiation in cardiac muscle and functional implications;

Figures 5A-I show photographs of a tissue sections from a MI induced mouse depicting regenerating myocardium in the area of the MI which has been injected with Lin⁻ c-kit^{POS} cells (**Figure 5A** is stained to show the presence of EGFP (green). Magnification is 300X. **Figure 5B** is stained to show the presence of α-smooth muscle actin in arterioles (red). Magnification is 300X. **Figure 5C** is stained
 15 to show the presence of both EGFP and α-smooth muscle actin (yellow-red), as well as PI-stained nuclei (blue). Magnification is 300X. **Figures 5D-F** and **G-I** depict the presence of MEF2 and Csx/Nkx2.5 in cardiac myosin positive cells. **Figure 5D** shows PI-stained nuclei (blue). Magnification is 300X. **Figure 5E** is stained to show MEF2 and Csx/Nkx2.5 labeling (green). Magnification is 300X. **Figure 5F** is
 20 stained to show cardiac myosin (red), as well as MEF2 or Csx/Nkx2.5 with PI (bright fluorescence in nuclei). Magnification is 300X. **Figure 5G** shows PI-stained nuclei (blue). Magnification is 300X. **Figure 5H** is stained to show MEF2 and Csx/Nkx2.5 labeling (green). Magnification is 300X. **Figure 5I** is stained to show cardiac myosin (red), as well as MEF2 or Csx/Nkx2.5 with PI (bright fluorescence
 25 in nuclei). Magnification is 300X);

Figure 6 (Figures 6A-F) shows photographs of tissue sections from MI induced mice, showing regenerating myocardium in the area of the MI injected with Lin⁻ c-kit^{POS} cells (**Figures 6A-C** show tissue which has been incubated in the presence of antibodies to BrdU. **Figure 6A** has been stained to show PI-labeled

nuclei (blue). Magnification is 900X. **Figure 6B** has been stained to show BrdU- and Ki67-labeled nuclei (green). Magnification is 900X. **Figure 6C** has been stained to show the presence of α -sarcomeric actin (red). Magnification is 900X. **Figures 6D-F** shows tissue that has been incubated in the presence of antibodies to Ki67. **Figure 6D** has been stained to show PI-labeled nuclei (blue). Magnification is 500X. **Figure 6E** has been stained to show BrdU- and Ki67-labeled nuclei (green). Magnification is 500X. **Figure 6F** has been stained to show the presence of α -smooth muscle actin (red). Magnification is 500X. Bright fluorescence: combination of PI with BrdU (C) or Ki67 (F));

Figure 7 (Figures 7A-C) shows photographs of tissue sections from MI induced mice, showing the area of MI injected with $\text{Lin}^- \text{c-kit}^{\text{POS}}$ cells (Depicted are the border zone, viable myocardium (VM) and the new band (NB) of myocardium separated by an area of infarcted non-repairing tissue (arrows). **Figure 7A** is stained to show the presence of EGFP (green). Magnification is 280X. **Figure 7B** is stained to show the presence of cardiac myosin (red). Magnification is 280X. **Figure 7C** is stained to show the presence of both EGFP and myosin (red-green), as well as PI-stained nuclei (blue). Magnification is 280X);

Figure 8 (Figures 8A-F) shows photographs of tissue sections from MI induced mice, showing regenerating myocardium in the area of MI injected with $\text{Lin}^- \text{c-kit}^{\text{POS}}$ cells (**Figure 8A** is stained to show the presence of EGFP (green). Magnification is 650X. **Figure 8B** is stained to show the presence of cardiac myosin (red). Magnification is 650X. **Figure 8C** is stained to show both the presence of EGFP and myosin (yellow), as well as PI-stained nuclei (blue). Magnification is 650X. **Figure 8D** is stained to show the presence of EGFP (green). Magnification is 650X. **Figure 8E** is stained to show the presence of α -smooth muscle actin in arterioles (red). Magnification is 650X. **Figure 8F** is stained to show the presence of both EGFP and α -smooth muscle actin (yellow-red) as well as PI-stained nuclei (blue). Magnification is 650X);

Figure 9 (Figures 9A-C) shows photographs of tissue sections from MI induced mice, showing the area of MI injected with $\text{Lin}^- \text{c-kit}^{\text{POS}}$ cells and showing regenerating myocardium (arrowheads). (**Figure 9A** is stained to show the presence of cardiac myosin (red) Magnification is 400X. **Figure 9B** is stained to show the presence of the Y chromosome (green). Magnification is 400X. **Figure 9C** is stained to show both the presence of the Y chromosome (light blue) and PI-labeled nuclei (dark blue). Note the lack of Y chromosome in infarcted tissue (IT) in subendocardium and spared myocytes (SM) in subepicardium. Magnification is 400X);

Figure 10 (Figures 10A-C) shows photographs of tissue sections from MI induced mice, showing GATA-4 in cardiac myosin positive cells (**Figure 10A** shows PI-stained nuclei (blue). Magnification is 650X. **Figure 10B** shows the presence of GATA-4 labeling (green). Magnification is 650X. **Figure 10C** is stained to show cardiac myosin (red) in combination with GATA-4 and PI (bright fluorescence in nuclei). Magnification is 650X);

Figure 11 (Figure 11A-D) shows photograph of tissue sections from a MI induced mouse (**Figure 11A** shows the border zone between the infarcted tissue and the surviving tissue. Magnification is 500X. **Figure 11B** shows regenerating myocardium. Magnification is 800X. **Figure 11C** is stained to show the presence of connexin 43 (yellow-green), and the contacts between myocytes are shown by arrows. Magnification is 800X. **Figure 11D** is stained to show both α -sarcomeric actin (red) and PI-stained nuclei (blue). Magnification is 800X);

Figure 12 (Figures 12A-B) shows photographs of tissue sections from a MI induced mouse showing the area of MI that was injected with $\text{Lin}^- \text{c-kit}^{\text{POS}}$ cells and now shows regenerating myocytes (**Figure 12A** is stained to show the presence of cardiac myosin (red) and PI-labeled nuclei (yellow-green). Magnification is 1,000. **Figure 12B** is the same as Figure 12A at a magnification of 700X);

Figures 13A-B show photographs of tissue sections from MI induced mice (**Figure 13A** shows a large infarct (MI) in a cytokine-treated mouse with forming

myocardium (arrowheads) (Magnification is 50X) at higher magnification (80X - adjacent panel). **Figure 13B** shows a MI in a non-treated mouse. Healing comprises the entire infarct (arrowheads) (Magnification is 50X). Scarring is seen at higher magnification (80X - adjacent panel). Red=cardiac myosin; yellow-

5 green=propidium iodide (PI) labeling of nuclei; blue-magenta=collagen types I and III);

Figure 13C shows a graph showing the mortality and myocardial regeneration in treated and untreated MI induced mice (Cytokine-treated infarcted mice, n=15; untreated infarcted mice, n=52. Log-rank test: $p<0.0001$);

10 **Figure 14** shows a graph showing quantitative measurement of infarct size (Total number of myocytes in the left ventricular free wall (LVFW) of sham-operated (SO, n=9), infarcted non-treated (MI, n=9) and cytokine-treated (MI-C, n=11) mice at sacrifice, 27 days after infarction or sham operation. The percentage of myocytes lost equals infarct size. $X\pm SD$, $*p<0.05$ vs SO);

15 **Figures 15A-C** show graphs comparing aspects of myocardial infarction, cardiac anatomy and function (**Figures 15 A-C** depict LV dimensions at sacrifice, 27 days after surgery; sham-operated (SO, n=9), non-treated infarcted (MI, n=9) and cytokine-treated infarcted (MI-C, n=10));

Figure 15D shows EF by echocardiography; (SO, n=9; MI, n=9; and MI-C, 20 n=9);

Figures 15E-M show M-mode echocardiograms of SO (e-g), MI (h-j) and MI-C (k-m) (Newly formed contracting myocardium (arrows));

Figure 15N shows a graph showing wall stress; SO (n=9), MI (n=8) and MI-C (n=9) (Results are mean \pm SD. $***p<0.05$ vs SO and MI, respectively);

25 **Figures 16A-G** show grafts depicting aspects of myocardial infarction, cardiac anatomy and ventricular function (**Figures 16A-D** show echocardiographic LVESD (a), LVEDD (b), PWST (c) and PWDT (d) in SO (n=9), MI (n=9) and MI-C (n=9). **Figures 16E-G** show mural thickness (e), chamber diameter (f) and

longitudinal axis (g) measured anatomically at sacrifice in SO (n=9), MI (n=9) and MI-C (n=10). ***p<0.05 vs SO and MI, respectively;

Figures 16H-P show two dimensional (2D) images and M-mode tracings of SO (h-j), MI (k-m) and MI-C (n-p);

5 **Figure 17 (Figures 17A-D)** shows graphs depicting aspects of ventricular function (**Figure 17A-D** show LV hemodynamics in anesthetized mice at sacrifice, 27 days after infarction or sham operation; SO (n=9), MI (n=9) and MI-C (n=10). For symbols and statistics, see also Figure 13);

Figure 18A-E shows graphs of aspects of myocardial regeneration (**Figure**
10 **18A** classifies the cells in the tissue as remaining viable (Re), lost (Lo) and newly formed (Fo) myocardium in LVFW at 27 days in MI and MI-C; SO, myocardium without infarct. **Figure 18B** shows the amount of cellular hypertrophy in spared myocardium. **Figure 18C** shows cell proliferation in the regenerating myocardium. Myocytes (M), EC and SMC labeled by BrdU and Ki67; n=11. ***p<0.05 vs M and
15 EC. **Figures 18D-E** depict the volume, number (n=11) and class distribution (bucket size, 100 μm^3 ; n=4,400) of myocytes within the formed myocardium;

Figures 18F-H show photographs of tissue sections from MI induced mice depicting arterioles with TER-119 labeled erythrocyte membrane (green fluorescence); blue fluorescence=PI staining of nuclei; red fluorescence= α -smooth
20 muscle actin in SMC (**Figure 18F** is magnified at 800X. **Figures 18G-H** are magnified at 1,200X);

Figure 19 (Figures 19A-D) shows photographs of tissue sections from MI induced mice that were incubated with antibodies to Ki67 (A,B) and BrdU (C,D) (**Figure 19A** shows labeling of myocytes by cardiac myosin. Bright fluorescence
25 of nuclei reflects the combination of PI and Ki67. Magnification is 800X. **Figure 19B** shows labeling of SMC by α -smooth muscle actin. Bright fluorescence of nuclei reflects the combination of PI and Ki67. Magnification is 1,200X. **Figure 19C** shows labeling of SMC by α -smooth muscle actin. Bright fluorescence of nuclei reflects the combination of PI and BrdU. Magnification is 1,200X. **Figure**

19D shows labeling of EC in the forming myocardium by factor VIII. Bright fluorescence of nuclei reflects the combination of PI and BrdU. Magnification is 1,600X;

Figure 20 (Figures 20A-F) shows photographs of tissue sections from MI induced mice showing markers of differentiating cardiac cells (Figure 20A is stained to show labeling of myocytes by nestin (yellow)). Red fluorescence indicates cardiac myosin. Magnification is 1,200X. Figure 20 B is stained to show labeling of desmin (red). Magnification is 800X. Figure 20C is stained to show labeling of connexin 43 (green). Red fluorescence indicates cardiac myosin. Magnification is 1,400X. Figure 20D shows VE-cadherin and yellow-green fluorescence reflects labeling of EC by flk-1 (arrows). Magnification is 1,800X. Figure 20E shows red fluorescence indicating factor VIII in EC and and yellow-green fluorescence reflects labeling of EC by flk-1 (arrows). Magnification is 1,200X. Figure 20F shows green fluorescence labeling of SMC cytoplasm by flk-1 and endothelial lining labeled by flk-1. Red fluorescence indicates α -smooth muscle actin. Blue fluorescence indicates PI labeling of nuclei. Magnification is 800X; and

Figure 21A-C show tissue sections from MI induced mice (Figure 21A uses bright fluorescence to depict the combination of PI labeling of nuclei with Csx/Nkx2.5. Magnification is 1,400X. Figure 21B uses bright fluorescence to depict the combination of PI labeling of nuclei with GATA-4. Magnification is 1,200X. Figure 21C uses bright fluorescence to depict the combination of PI labeling of nuclei with MEF2. Magnification is 1,200X (Red fluorescence shows cardiac myosin antibody staining and blue fluorescence depicts PI labeling of nuclei. The fraction of myocyte nuclei labeled by Csx/Nkx2.5, GATA-4 and MEF2 was $63 \pm 5\%$ (nuclei sampled=2,790; n=11), $94 \pm 9\%$ (nuclei sampled=2,810; n=11) and $85 \pm 14\%$ (nuclei sampled= 3,090; n=11), respectively).

DETAILED DESCRIPTION

The present invention provides methods and/or pharmaceutical composition comprising a therapeutically effective amount of somatic stem cells alone or in combination with a cytokine selected from the group consisting of stem cell factor (SCF), granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), stromal cell-derived factor-1, steel factor, vascular endothelial growth factor, macrophage colony stimulating factor, granulocyte-macrophage stimulating factor or Interleukin-3 or any cytokine capable of the stimulating and/or mobilizing stem cells. Cytokines may be administered alone or in combination or with any other cytokine capable of: the stimulation and/or mobilization of stem cells; the maintenance of early and late hematopoiesis (see below); the activation of monocytes (see below), macrophage/monocyte proliferation; differentiation, motility and survival (see below) and a pharmaceutically acceptable carrier, diluent or excipient (including combinations thereof).

The cytokines in the pharmaceutical composition of the present invention may also include mediators known to be involved in the maintenance of early and late hematopoiesis such as IL-1 alpha and IL-1 beta, IL-6, IL-7, IL-8, IL-11 and IL-13; colony-stimulating factors, thrombopoietin, erythropoietin, stem cell factor, fit 3-ligand, hepatocyte cell growth factor, tumor necrosis factor alpha, leukemia inhibitory factor, transforming growth factors beta 1 and beta 3; and macrophage inflammatory protein 1 alpha), angiogenic factors (fibroblast growth factors 1 and 2, vascular endothelial growth factor) and mediators whose usual target (and source) is the connective tissue-forming cells (platelet-derived growth factor A, epidermal growth factor, transforming growth factors alpha and beta 2, oncostatin M and insulin-like growth factor-1), or neuronal cells (nerve growth factor) (Sensebe, L., et al., *Stem Cells* 1997; 15:133-43), VEGF polypeptides that are present in platelets and megacaryocytes (Wartiovaara, U., et al., *Thromb Haemost* 1998; 80:171-5; Mohle, R., *Proc Natl Acad Sci USA* 1997; 94:663-8) HIF-1, a potent transcription factor that binds to and stimulates the promoter of several genes involved in responses to hypoxia, endothelial PAS domain protein 1 (EPAS 1), monocyte-derived cytokines for enhancing collateral function such as monocyte chemotactic protein-1 (MCP-1).

In a preferred aspect, the pharmaceutical composition of the present invention is delivered via injection. These routes for administration (delivery) include, but are not limited to subcutaneous or parenteral including intravenous, intraarterial, intramuscular, intraperitoneal, intramyocardial, transendocardial, trans-epicardial, intranasal administration as well as intrathecal, and infusion techniques. Hence, preferably the pharmaceutical composition is in a form that is suitable for injection.

When administering a therapeutic of the present invention parenterally, it will generally be formulated in a unit dosage injectable form (solution, suspension, emulsion). The pharmaceutical formulations suitable for injection include sterile aqueous solutions or dispersions and sterile powders for reconstitution into sterile injectable solutions or dispersions. The carrier can be a solvent or dispersing medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils.

Proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Nonaqueous vehicles such as cottonseed oil, sesame oil, olive oil, soybean oil, corn oil, sunflower oil, or peanut oil and esters, such as isopropyl myristate, may also be used as solvent systems for compound compositions

Additionally, various additives which enhance the stability, sterility, and isotonicity of the compositions, including antimicrobial preservatives, antioxidants, chelating agents, and buffers, can be added. Prevention of the action of microorganisms can be ensured by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, and the like. In many cases, it will be desirable to include isotonic agents, for example, sugars, sodium chloride, and the like. Prolonged absorption of the injectable pharmaceutical form can be brought about by the use of agents delaying absorption, for example, aluminum monostearate and gelatin. According to the present invention, however, any vehicle, diluent, or additive used would have to be compatible with the compounds.

Sterile injectable solutions can be prepared by incorporating the compounds utilized in practicing the present invention in the required amount of the appropriate solvent with various amounts of the other ingredients, as desired.

The pharmaceutical composition of the present invention, e.g., comprising a
5 therapeutic compound, can be administered to the patient in an injectable formulation containing any compatible carrier, such as various vehicles, adjuvants, additives, and diluents; or the compounds utilized in the present invention can be administered parenterally to the patient in the form of slow-release subcutaneous implants or targeted delivery systems such as monoclonal antibodies, iontophoretic,
10 polymer matrices, liposomes, and microspheres.

The pharmaceutical composition utilized in the present invention can be administered orally to the patient. Conventional methods such as administering the compounds in tablets, suspensions, solutions, emulsions, capsules, powders, syrups and the like are usable. Known techniques which deliver the compound orally or
15 intravenously and retain the biological activity are preferred.

In one embodiment, a composition of the present invention can be administered initially, and thereafter maintained by further administration. For instance, a composition of the invention can be administered in one type of composition and thereafter further administered in a different or the same type of
20 composition. For example, a composition of the invention can be administered by intravenous injection to bring blood levels to a suitable level. The patient's levels are then maintained by an oral dosage form, although other forms of administration, dependent upon the patient's condition, can be used.

It is noted that humans are treated generally longer than the mice or other
25 experimental animals which treatment has a length proportional to the length of the disease process and drug effectiveness. The doses may be single doses or multiple doses over a period of several days, but single doses are preferred. Thus, one can scale up from animal experiments, e.g., rats, mice, and the like, to humans, by techniques from this disclosure and documents cited herein and the knowledge in the
30 art, without undue experimentation.

The treatment generally has a length proportional to the length of the disease process and drug effectiveness and the patient being treated.

The quantity of the pharmaceutical composition to be administered will vary for the patient being treated. In a preferred embodiment, $2 \times 10^4 - 1 \times 10^5$ stem cells and 50-500 $\mu\text{g/kg}$ per day of a cytokine were administered to the patient. While there would be an obvious size difference between the hearts of a mouse and a human, it is possible that $2 \times 10^4 - 1 \times 10^5$ stem cells would be sufficient in a human as well. However, the precise determination of what would be considered an effective dose may be based on factors individual to each patient, including their size, age, size of the infarct, and amount of time since damage. Therefore, dosages can be readily ascertained by those skilled in the art from this disclosure and the knowledge in the art. Thus, the skilled artisan can readily determine the amount of compound and optional additives, vehicles, and/or carrier in compositions and to be administered in methods of the invention. Typically, any additives (in addition to the active stem cell(s) and/or cytokine(s)) are present in an amount of 0.001 to 50 wt% solution in phosphate buffered saline, and the active ingredient is present in the order of micrograms to milligrams, such as about 0.0001 to about 5 wt%, preferably about 0.0001 to about 1 wt%, most preferably about 0.0001 to about 0.05 wt% or about 0.001 to about 20 wt%, preferably about 0.01 to about 10 wt%, and most preferably about 0.05 to about 5 wt%. Of course, for any composition to be administered to an animal or human, and for any particular method of administration, it is preferred to determine therefore: toxicity, such as by determining the lethal dose (LD) and LD_{50} in a suitable animal model e.g., rodent such as mouse; and, the dosage of the composition(s), concentration of components therein and timing of administering the composition(s), which elicit a suitable response. Such determinations do not require undue experimentation from the knowledge of the skilled artisan, this disclosure and the documents cited herein. And, the time for sequential administrations can be ascertained without undue experimentation.

Examples of compositions comprising a therapeutic of the invention include liquid preparations for orifice, e.g., oral, nasal, anal, vaginal, peroral, intragastric, mucosal (e.g., perlingual, alveolar, gingival, olfactory or respiratory mucosa) etc., administration such as suspensions, syrups or elixirs; and, preparations for parenteral, subcutaneous, intradermal, intramuscular or intravenous administration (e.g., injectable administration), such as sterile suspensions or emulsions. Such

compositions may be in admixture with a suitable carrier, diluent, or excipient such as sterile water, physiological saline, glucose or the like. The compositions can also be lyophilized. The compositions can contain auxiliary substances such as wetting or emulsifying agents, pH buffering agents, gelling or viscosity enhancing additives, preservatives, flavoring agents, colors, and the like, depending upon the route of administration and the preparation desired. Standard texts, such as "REMINGTON'S PHARMACEUTICAL SCIENCE", 17th edition, 1985, incorporated herein by reference, may be consulted to prepare suitable preparations, without undue experimentation.

Compositions of the invention, are conveniently provided as liquid preparations, e.g., isotonic aqueous solutions, suspensions, emulsions or viscous compositions which may be buffered to a selected pH. If digestive tract absorption is preferred, compositions of the invention can be in the "solid" form of pills, tablets, capsules, caplets and the like, including "solid" preparations which are time-released or which have a liquid filling, e.g., gelatin covered liquid, whereby the gelatin is dissolved in the stomach for delivery to the gut. If nasal or respiratory (mucosal) administration is desired, compositions may be in a form and dispensed by a squeeze spray dispenser, pump dispenser or aerosol dispenser. Aerosols are usually under pressure by means of a hydrocarbon. Pump dispensers can preferably dispense a metered dose or, a dose having a particular particle size.

Compositions of the invention can contain pharmaceutically acceptable flavors and/or colors for rendering them more appealing, especially if they are administered orally. The viscous compositions may be in the form of gels, lotions, ointments, creams and the like (e.g., for transdermal administration) and will typically contain a sufficient amount of a thickening agent so that the viscosity is from about 2500 to 6500 cps, although more viscous compositions, even up to 10,000 cps may be employed. Viscous compositions have a viscosity preferably of 2500 to 5000 cps, since above that range they become more difficult to administer. However, above that range, the compositions can approach solid or gelatin forms which are then easily administered as a swallowed pill for oral ingestion.

Liquid preparations are normally easier to prepare than gels, other viscous compositions, and solid compositions. Additionally, liquid compositions are somewhat more convenient to administer, especially by injection or orally. Viscous

compositions, on the other hand, can be formulated within the appropriate viscosity range to provide longer contact periods with mucosa, such as the lining of the stomach or nasal mucosa.

Obviously, the choice of suitable carriers and other additives will depend on the exact route of administration and the nature of the particular dosage form, e.g., liquid dosage form (e.g., whether the composition is to be formulated into a solution, a suspension, gel or another liquid form), or solid dosage form (e.g., whether the composition is to be formulated into a pill, tablet, capsule, caplet, time release form or liquid-filled form).

Solutions, suspensions and gels normally contain a major amount of water (preferably purified water) in addition to the active compound. Minor amounts of other ingredients such as pH adjusters (e.g., a base such as NaOH), emulsifiers or dispersing agents, buffering agents, preservatives, wetting agents, jelling agents, (e.g., methylcellulose), colors and/or flavors may also be present. The compositions can be isotonic, i.e., they can have the same osmotic pressure as blood and lacrimal fluid.

The desired isotonicity of the compositions of this invention may be accomplished using sodium chloride, or other pharmaceutically acceptable agents such as dextrose, boric acid, sodium tartrate, propylene glycol or other inorganic or organic solutes. Sodium chloride is preferred particularly for buffers containing sodium ions.

Viscosity of the compositions may be maintained at the selected level using a pharmaceutically acceptable thickening agent. Methylcellulose is preferred because it is readily and economically available and is easy to work with. Other suitable thickening agents include, for example, xanthan gum, carboxymethyl cellulose, hydroxypropyl cellulose, carbomer, and the like. The preferred concentration of the thickener will depend upon the agent selected. The important point is to use an amount which will achieve the selected viscosity. Viscous compositions are normally prepared from solutions by the addition of such thickening agents.

A pharmaceutically acceptable preservative can be employed to increase the shelf-life of the compositions. Benzyl alcohol may be suitable, although a variety of preservatives including, for example, parabens, thimerosal, chlorobutanol, or benzalkonium chloride may also be employed. A suitable concentration of the

preservative will be from 0.02% to 2% based on the total weight although there may be appreciable variation depending upon the agent selected.

Those skilled in the art will recognize that the components of the compositions should be selected to be chemically inert with respect to the active compound. This will present no problem to those skilled in chemical and pharmaceutical principles, or problems can be readily avoided by reference to standard texts or by simple experiments (not involving undue experimentation), from this disclosure and the documents cited herein.

The inventive compositions of this invention are prepared by mixing the ingredients following generally accepted procedures. For example the selected components may be simply mixed in a blender, or other standard device to produce a concentrated mixture which may then be adjusted to the final concentration and viscosity by the addition of water or thickening agent and possibly a buffer to control pH or an additional solute to control tonicity. Generally the pH may be from about 3 to 7.5. Compositions can be administered in dosages and by techniques well known to those skilled in the medical and veterinary arts taking into consideration such factors as the age, sex, weight, and condition of the particular patient, and the composition form used for administration (e.g., solid vs. liquid). Dosages for humans or other mammals can be determined without undue experimentation by the skilled artisan, from this disclosure, the documents cited herein, and the knowledge in the art.

Suitable regimes for initial administration and further doses or for sequential administrations also are variable, may include an initial administration followed by subsequent administrations; but nonetheless, may be ascertained by the skilled artisan, from this disclosure, the documents cited herein, and the knowledge in the art.

The pharmaceutical compositions of the present invention are used to treat cardiovascular diseases, including, but not limited to, atherosclerosis, ischemia, hypertension, restenosis, angina pectoris, rheumatic heart disease, congenital cardiovascular defects and arterial inflammation and other diseases of the arteries, arterioles and capillaries or related complaint. Accordingly, the invention involves the administration of stem cells as herein discussed, alone or in combination with one or more cytokine, as herein discussed, for the treatment or prevention of any one

or more of these conditions or other conditions involving weakness in the heart, as well as compositions for such treatment or prevention, use of stem cells as herein discussed, alone or in combination with one or more cytokine, as herein discussed, for formulating such compositions, and kits involving stem cells as herein discussed, alone or in combination with one or more cytokine, as herein discussed, for preparing such compositions and/or for such treatment, or prevention. And, advantageous routes of administration involves those best suited for treating these conditions, such as via injection, including, but are not limited to subcutaneous or parenteral including intravenous, intraarterial, intramuscular, intraperitoneal, intramyocardial, transendocardial, trans-epicardial, intranasal administration as well as intrathecal, and infusion techniques.

The pharmaceutical compositions of the present invention may be used as therapeutic agents – i.e. in therapy applications. As herein, the terms “treatment” and “therapy” include curative effects, alleviation effects, and prophylactic effects.

As used herein, “patient” may encompass any vertebrate including but not limited to humans, mammals, reptiles, amphibians and fish. However, advantageously, the patient is a mammal such as a human, or an animal mammal such as a domesticated mammal, e.g., dog, cat, horse, and the like, or production mammal, e.g., cow, sheep, pig, and the like

As used herein “somatic stem cell” or “stem cell” or “hematopoietic cell” refers to either autologous or allogenic stem cells, which may be obtained from the bone marrow, peripheral blood, or other source.

As used herein, “adult” stem cells refers to stem cells that are not embryonic in origin nor derived from embryos or fetal tissue.

As used herein “recently damaged myocardium” refers to myocardium which has been damaged within one week of treatment being started. In a preferred embodiment, the myocardium has been damaged within three days of the start of treatment. In a further preferred embodiment, the myocardium has been damaged within 12 hours of the start of treatment. It is advantageous to employ stem cells alone or in combination with cytokine(s) as herein disclosed to a recently damaged myocardium.

As used herein “damaged myocardium” refers to myocardial cells which have been exposed to ischemic conditions. These ischemic conditions may be

caused by a myocardial infarction, or other cardiovascular disease or related complaint. The lack of oxygen causes the death of the cells in the surrounding area, leaving an infarct, which will eventually scar.

As used herein, "home" refers to the attraction and mobilization of somatic stem cells towards damaged myocardium and/or myocardial cells.

As used herein, "assemble" refers to the assembly of differentiated somatic stem cells into functional structures i.e., myocardium and/or myocardial cells, coronary arteries, arterioles, and capillaries etc. This assembly provides functionality to the differentiated myocardium and/or myocardial cells, coronary arteries, arterioles and capillaries.

Thus, the invention involves the use of somatic stem cells. These are present in animals in small amounts, but methods of collecting stem cells are known to those skilled in the art.

In another aspect of the invention, the stem cells are selected to be lineage negative. The term "lineage negative" is known to one skilled in the art as meaning the cell does not express antigens characteristic of specific cell lineages.

Advantageously, the lineage negative stem cells are selected to be *c-kit* positive. The term "c-kit" is known to one skilled in the art as being a receptor which is known to be present on the surface of stem cells, and which is routinely utilized in the process of identifying and separating stem cells from other surrounding cells.

The invention further involves a therapeutically effective dose or amount of stem cells applied to the heart. An effective dose is an amount sufficient to effect a beneficial or desired clinical result. Said dose could be administered in one or more administrations. In the examples that follow, $2 \times 10^4 - 1 \times 10^5$ stem cells were administered in the mouse model. While there would be an obvious size difference between the hearts of a mouse and a human, it is possible that this range of stem cells would be sufficient in a human as well. However, the precise determination of what would be considered an effective dose may be based on factors individual to each patient, including their size, age, size of the infarct, and amount of time since damage. One skilled in the art, specifically a physician or cardiologist, would be able to determine the number of stem cells that would constitute an effective dose without undue experimentation.

In another aspect of the invention, the stem cells are delivered to the heart, specifically to the border area of the infarct. As one skilled in the art would be aware, the infarcted area is visible grossly, allowing this specific placement of stem cells to be possible.

5 The stem cells are advantageously administered by injection, specifically an intramyocardial injection. As one skilled in the art would be aware, this is the preferred method of delivery for stem cells as the heart is a functioning muscle. Injection of the stem cells into the heart ensures that they will not be lost due to the contracting movements of the heart.

10 In a further aspect of the invention, the stem cells are administered by injection transendocardially or trans-epicardially. This preferred embodiment allows the stem cells to penetrate the protective surrounding membrane, necessitated by the embodiment in which the cells are injected intramyocardially.

15 A preferred embodiment of the invention includes use of a catheter-based approach to deliver the trans-endocardial injection. The use of a catheter precludes more invasive methods of delivery wherein the opening of the chest cavity would be necessitated. As one skilled in the art is aware, optimum time of recovery would be allowed by the more minimally invasive procedure, which as outlined here, includes a catheter approach.

20 Further embodiments of the invention require the stem cells to migrate into the infarcted region and differentiate into myocytes, smooth muscle cells, and endothelial cells. It is known in the art that these types of cells must be present to restore both structural and functional integrity. Other approaches to repairing infarcted or ischemic tissue have involved the implantation of these cells directly
25 into the heart, or as cultured grafts, such as in U.S. Patent No. 6,110,459, and 6,099,832.

30 Another embodiment of the invention includes the proliferation of the differentiated cells and the formation of the cells into cardiac structures including coronary arteries, arterioles, capillaries, and myocardium. As one skilled in the art is aware, all of these structures are essential for proper function in the heart. It has been shown in the literature that implantation of cells including endothelial cells and smooth muscle cells will allow for the implanted cells to live within the infarcted region, however they do not form the necessary structures to enable the heart to

regain full functionality. The ability to restore both functional and structural integrity is yet another aspect of this invention.

Another aspect of the invention relates to the administration of a cytokine. This cytokine may be chosen from a group of cytokines, or may include combinations of cytokines. Stem cell factor (SCF) and granulocyte-colony stimulating factor (G-CSF) are known by those skilled in the art as stimulating factors which cause the mobilization of stem cells into the blood stream (Bianco et al, 2001, Clutterbuck, 1997, Kronenwett et al, 2000, LaIuppa et al, 1997, Patchen et al, 1998). Stromal cell-derived factor-1 has been shown to stimulate stem cell mobilization chemotactically, while steel factor has both chemotactic and chemokinetic properties (Caceres-Cortes et al, 2001, Jo et al, 2000, Kim and Broxmeyer, 1998, Ikuta et al, 1991). Vascular endothelial growth factor has been surmised to engage a paracrine loop that helps facilitate migration during mobilization (Bautz et al, 2000, Janowska-Wieczorek et al, 2001). Macrophage colony stimulating factor and granulocyte-macrophage stimulating factor have been shown to function in the same manner of SCF and G-CSF, by stimulating mobilization of stem cells. Interleukin-3 has also been shown to stimulate mobilization of stem cells, and is especially potent in combination with other cytokines.

The cytokine can be administered via a vector that expresses the cytokine *in vivo*. A vector for *in vivo* expression can be a vector or cells or an expression system as cited in any document incorporated herein by reference or used in the art, such as a viral vector, e.g., an adenovirus, poxvirus (such as vaccinia, canarypox virus, MVA, NYVAC, ALVAC, and the like), lentivirus or a DNA plasmid vector; and, the cytokine can also be from *in vitro* expression via such a vector or cells or expression system or others such as a baculovirus expression system, bacterial vectors such as *E. coli*, and mammalian cells such as CHO cells. *See, e.g.*, U.S. Patent Nos. 6,265,189, 6,130,066, 6,004,777, 5,990,091, 5,942,235, 5,833,975. The cytokine compositions may lend themselves to administration by routes outside of those stated to be advantageous or preferred for stem cell preparations; but, cytokine compositions may also be advantageously administered by routes stated to be advantageous or preferred for stem cell preparations.

A further aspect of the invention involves administration of a therapeutically effective dose or amount of a cytokine. An effective dose is an amount sufficient to effect a beneficial or desired clinical result. Said dose could be administered in one or more administrations. In a preferred embodiment, the dose would be given over
5 the course of about two or three days following the beginning of treatment. However, the precise determination of what would be considered an effective dose may be based on factors individual to each patient, including their size, age, size of the infarct, the cytokine or combination of cytokines being administered, and amount of time since damage. One skilled in the art, specifically a physician or
10 cardiologist, would be able to determine a sufficient amount of cytokine that would constitute an effective dose without being subjected to undue experimentation.

The invention also involves the administration of the therapeutically effective dose or amount of a cytokine being delivered by injection, specifically subcutaneously or intravenously. A person skilled in the art will be aware that
15 subcutaneous injection or intravenous delivery are extremely common and offer an effective method of delivering the specific dose in a manner which allows for timely uptake and circulation in the blood stream.

A further aspect of the invention includes the administered cytokine stimulating the patient's stem cells and causing mobilization into the blood stream.
20 As mentioned previously, the given cytokines are well-known to one skilled in the art for their ability to promote said mobilization.

Advantageously, once the stem cells have mobilized into the bloodstream, they home to the damaged area of the heart, as will become clear through the following examples.

25 Further embodiments of the invention involve the stem cells migrating into the infarcted region and differentiating into myocytes, smooth muscle cells, and endothelial cells. It is known in the art that these types of cells must be present to restore both structural and functional integrity.

Another embodiment of the invention includes the proliferation of the
30 differentiated cells and the formation of the cells into cardiac structures including coronary arteries, arterioles, capillaries, and myocardium. As one skilled in the art is aware, all of these structures are important for proper function in the heart. It has been shown in the literature that implantation of cells including endothelial cells and

smooth muscle cells will allow for the implanted cells to live within the infarcted region, however they do not form the necessary structures to enable the heart to regain full functionality. The ability to restore both functional and structural integrity or better functional and structural integrity than previously achieved in the art is yet another aspect of this invention.

It is preferred in the practice of the invention to utilize both the administration of stem cells and that of a cytokine to ensure the most effective method of repairing damaged myocardium.

Stem cells employed in the invention are advantageously selected to be lineage negative. The term "lineage negative" is known to one skilled in the art as meaning the cell does not express antigens characteristic of specific cell lineages. And, it is advantageous that the lineage negative stem cells are selected to be *c-kit* positive. The term "*c-kit*" is known to one skilled in the art as being a receptor which is known to be present on the surface of stem cells, and which is routinely utilized in the process of identifying and separating stem cells from other surrounding cells.

In certain embodiments, a therapeutically effective dose of stem cells is applied, delivered, or administered to the heart or implanted into the heart. An effective dose or amount is an amount sufficient to effect a beneficial or desired clinical result. Said dose could be administered in one or more administrations. In the examples that follow, $2 \times 10^4 - 1 \times 10^5$ stem cells were administered in the mouse model. While there would be an obvious size difference between the hearts of a mouse and a human, it is possible that $2 \times 10^4 - 1 \times 10^5$ stem cells would be sufficient in a human as well. However, the precise determination of what would be considered an effective dose may be based on factors individual to each patient, including their size, age, size of the infarct, and amount of time since damage. One skilled in the art, specifically a physician or cardiologist, would be able to determine the number and type (or types) of stem cells which would constitute an effective dose without being subjected to undue experimentation, from this disclosure and the knowledge in the art; and, in this regard and in general in regard to preparing formulations and administering formulations or components thereof, mention is made of the teachings in the Examples and that the skilled artisan can scale dosages, amounts and the like based on the weight of the patient to be treated in comparison

to the weight of any animal employed in the Examples. The stem cells are advantageously bone marrow or are cardiac stem cells; and even more advantageously, the stem cells are adult bone marrow (hematopoietic stem cells) or adult cardiac stem cells or a combination thereof or a combination of cardiac stem cells such as adult cardiac stem cells and another type of stem cell such as another type of adult stem cells.

In another aspect of the invention, the stem cells are delivered to the heart, specifically to the border area of the infarct. As one skilled in the art would be aware, the infarcted area is visible grossly, allowing this specific placement of stem cells to be possible.

The stem cells are advantageously administered by injection, specifically an intramyocardial injection. As one skilled in the art would be aware, this is the preferred method of delivery for stem cells as the heart is a functioning muscle. Injection of the stem cells into the heart ensures that they will not be lost due to the contracting movements of the heart.

In other aspects of the invention, the stem cells are administered by injection transendocardially or trans-epicardially. This preferred embodiment allows the stem cells to penetrate the protective surrounding membrane, necessitated by the embodiment in which the cells are injected intramyocardially.

A preferred embodiment of the invention includes use of a catheter-based approach to deliver the trans-endocardial injection. The use of a catheter precludes more invasive methods of delivery wherein the opening of the chest cavity would be necessitated. As one skilled in the art is aware, optimum time of recovery would be allowed by the more minimally invasive procedure, which as outlined here, includes a catheter approach.

Embodiments of the invention can involve the administration of a cytokine. This cytokine may be chosen from a group of cytokines, or may include combinations of cytokines.

A further aspect of the invention involves administration of a therapeutically effective dose of a cytokine. An effective dose or amount is an amount sufficient to effect a beneficial or desired clinical result. Said dose could be administered in one or more administrations. In a preferred embodiment, the dose would be given over the course of about two or three days following the beginning of treatment.

However, the precise determination of what would be considered an effective dose may be based on factors individual to each patient, including their size, age, size of the infarct, the cytokine or combination of cytokines being administered, and amount of time since damage. One skilled in the art, specifically a physician or cardiologist, would be able to determine a sufficient amount of cytokine that would constitute an effective dose without being subjected to undue experimentation, especially in view of the disclosure herein and the knowledge in the art.

The administration of the therapeutically effective dose of at least one cytokine is advantageously by injection, specifically subcutaneously or intravenously. A person skilled in the art will be aware that subcutaneous injection or intravenous delivery are extremely common and offer an effective method of delivering the specific dose in a manner which allows for timely uptake and circulation in the blood stream.

A further aspect of the invention includes the administered cytokine stimulating the patient's stem cells and causing mobilization into the blood stream. As mentioned previously, the given cytokines are well known to one skilled in the art for their ability to promote said mobilization. Again, once the stem cells have mobilized into the bloodstream, they home to the damaged area of the heart. Thus in certain embodiments, both the implanted stem cells and the mobilized stem cells migrate into the infarct region and differentiate into myocytes, smooth muscle cells, and endothelial cells. It is known in the art that these types of cells are advantageously present to restore both structural and functional integrity.

Another embodiment of the invention includes the proliferation of the differentiated cells and the formation of the cells into cardiac structures including coronary arteries, arterioles, capillaries, and myocardium. As one skilled in the art is aware, all of these structures are essential for proper function in the heart. It has been shown in the literature that implantation of cells including endothelial cells and smooth muscle cells will allow for the implanted cells to live within the infarcted region, however they do not form the necessary structures to enable the heart to regain full functionality. Cardiac structures can be generated *ex vivo* and then implanted in the form of a graft; with the implantation of the graft being alone or in combination with stem cells or stem cells and at least one cytokine as in this disclosure, e.g., advantageously adult or cardiac or hematopoietic stem cells such as

adult cardiac and/or adult hematopoietic stem cells or adult cardiac stem cells with another type of stem cell e.g. another type of adult stem cell. The means of generating and/or regenerating myocardium *ex vivo*, may incorporate somatic stem cells and heart tissue being cultured *in vitro*, optionally in the presence of a cytokine.

- 5 The somatic stem cells differentiate into myocytes, smooth muscle cells and endothelial cells, and proliferate *in vitro*, forming myocardial tissue and/or cells. These tissues and cells may assemble into cardiac structures including arteries, arterioles, capillaries, and myocardium. The tissue and/or cells formed *in vitro* may then be implanted into a patient, e.g. via a graft, to restore structural and functional
10 integrity.

Additionally or alternatively, the source of the tissue being grafted can be from other sources of tissue used in grafts of the heart.

- The restoration or some restoration of both functional and structural integrity of cardiac tissue – advantageously over that which has occurred previously - is yet
15 another aspect of this invention.

Accordingly, the invention comprehends, in further aspects, methods for preparing compositions such as pharmaceutical compositions including somatic stem cells and/or at least one cytokine, for instance, for use in inventive methods for treating cardiovascular disease or conditions or cardiac conditions.

- 20 The present invention is additionally described by way of the following, non-limiting examples, that provide a better understanding of the present invention and of its many advantages.

- All of the materials, reagents, chemicals, assays, cytokines, antibodies, and miscellaneous items referred to in the following examples are readily available to
25 the research community through commercial suppliers, including but not limited to, Genzyme, Invitrogen, Gibco BRL, Clonetics, Fisher Scientific, R&D Systems, MBL International Corporation, CN Biosciences Corporate, Sigma Aldrich, and CedarLane Laboratories, Limited.

- 30 For example,
stem cell factor is available under the name SCF (multiple forms of recombinant human, recombinant mouse, and antibodies to each),

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from R & D Systems (614 McKinley Place N.E., Minneapolis, MN 55413);

granulocyte-colony stimulating factor is available under the name G-CSF (multiple forms of recombinant human, recombinant mouse, and antibodies to each), from R & D Systems;

stem cell antibody-1 is available under the name SCA-1 from MBL International Corporation (200 Dexter Avenue, Suite D, Watertown, MA 02472);

multidrug resistant antibody is available under the name Anti-MDR from CN Biosciences Corporate;

c-kit antibody is available under the name *c-kit* (Ab-1) Polyclonal Antibody from CN Biosciences Corporate (Affiliate of Merck KgaA, Darmstadt, Germany. Corporate headquarters located at 10394 Pacific Center Court, San Diego, CA 92121).

EXAMPLES

EXAMPLE 1: Hematopoietic Stem Cell (HSC) Repair of Infarcted Myocardium

A. Harvesting of Hematopoietic Stem Cells

Bone marrow was harvested from the femurs and tibias of male transgenic mice expressing enhanced green fluorescent protein (EGFP). After surgical removal of the femurs and tibias, the muscle was dissected and the upper and lower surface of the bone was cut on the surface to allow the collecting buffer to infiltrate the bone marrow. The fluid containing buffer and cells was collected in tubes such as 1.5 ml Eppendorf tubes. Bone marrow cells were suspended in PBS containing 5% fetal calf serum (FCS) and incubated on ice with rat anti-mouse monoclonal antibodies specific for the following hematopoietic lineages: CD4 and CD8 (T-lymphocytes), B-220 (B-lymphocytes), Mac-1 (macrophages), GR-1 (granulocytes) (Caltag Laboratories) and TER-119 (erythrocytes) (Pharmingen). Cells were then rinsed in PBS and incubated for 30 minutes with magnetic beads coated with goat anti-rat immunoglobulin (Polysciences Inc.). Lineage positive cells (Lin⁺) were removed by a biomagnet and lineage negative cells (Lin⁻) were stained with ACK-4-biotin (anti-*c-kit* mAb). Cells were rinsed in PBS, stained with streptavidin-conjugated phycoerythrin (SA-PE) (Caltag Labs.) and sorted by fluorescence activated cell

sorting (FACS) using a FACSVantage instrument (Becton Dickinson). Excitation of EGFP and ACK-4-biotin-SA-EP occurred at a wavelength of 488 nm. The Lin⁻ cells were sorted as *c-kit* positive (*c-kit*^{POS}) and *c-kit* negative (*c-kit*^{NEG}) with a 1-2 log difference in staining intensity (Figure 1). The *c-kit*^{POS} cells were suspended at 2 x 10⁴ to 1 x 10⁵ cells in 5 µl of PBS and the *c-kit*^{NEG} cells were suspended at a concentration of 1 x 10⁵ in 5 µl of PBS.

B. Induction of Myocardial Infarction in Mice

Myocardial infarction was induced in female C57BL/6 mice at 2 months of age as described by Li et al. (1997). Three to five hours after infarction, the thorax of the mice was reopened and 2.5 µl of PBS containing Lin⁻*c-kit*^{POS} cells were injected in the anterior and posterior aspects of the viable myocardium bordering the infarct (Figure 2). Infarcted mice, left uninjected or injected with Lin⁻*c-kit*^{NEG} cells, and sham-operated mice *i.e.*, mice where the chest cavity was opened but no infarction was induced, were used as controls. All animals were sacrificed 9 ± 2 days after surgery. Protocols were approved by institutional review board. Results are presented as mean ± SD. Significance between two measurements was determined by the Student's *t* test, and in multiple comparisons was evaluated by the Bonferroni method (Scholzen and Gerdes, 2000). P<0.05 was considered significant.

Injection of male Lin⁻*c-kit*^{POS} bone marrow cells in the peri-infarcted left ventricle of female mice resulted in myocardial regeneration. The peri-infarcted region is the region of viable myocardium bordering the infarct. Repair was obtained in 12 of 30 mice (40%). Failure to reconstitute infarcts was attributed to the difficulty of transplanting cells into tissue contracting at 600 beats per minute (bpm). However, an immunologic reaction to the histocompatibility antigen on the Y chromosome of the donor bone marrow cells could account for the lack of repair in some of the female recipients. Closely packed myocytes occupied 68±11% of the infarcted region and extended from the anterior to the posterior aspect of the ventricle (Figs. 2A-2D). New myocytes were not found in mice injected with Lin⁻*c-kit*^{NEG} cells (Fig. 2E).

C. Determination of Ventricular Function

Mice were anesthetized with chloral hydrate (400 mg/kg body weight, i.p.), and the right carotid artery was cannulated with a microtip pressure transducer (model SPR-671, Millar) for the measurements of left ventricular (LV) pressures and LV + and -dP/dt in the closed-chest preparation to determine whether

5 developing myocytes derived from the HSC transplant had an impact on function. Infarcted mice non-injected or injected with Lin⁻c-kit^{NEG} cells were combined in the statistics. In comparison with sham-operated groups, the infarcted groups exhibited indices of cardiac failure (Figure 3). In mice treated with Lin-c-kit^{POS} cells, LV end-diastolic pressure (LVEDP) was 36% lower, and developed pressure (LVDP) and

10 LV+ and -dP/dt were 32%, 40%, and 41% higher, respectively (Figure 4A).

D. Determination of Cell Proliferation and EGFP Detection

The abdominal aorta was cannulated, the heart was arrested in diastole by injection of cadmium chloride (CdCl₂), and the myocardium was perfused retrogradely with 10% buffered formalin. Three tissue sections, from the base to the

15 apex of the left ventricle, were stained with hematoxylin and eosin. At 9 ± 2 days after coronary occlusion, the infarcted portion of the ventricle was easily identifiable grossly and histologically (see Fig. 2A). The lengths of the endocardial and epicardial surfaces delimiting the infarcted region, and the endocardium and epicardium of the entire left ventricle were measured in each section. Subsequently,

20 their quotients were computed to yield the average infarct size in each case. This was accomplished at 4X magnification utilizing an image analyzer connected to a microscope. The fraction of endocardial and epicardial circumference delimiting the infarcted area (Pfeffer and Braunwald, 1990; Li et al., 1997) did not differ in untreated mice, 78±18% (n=8) and in mice treated with Lin⁻c-kit^{POS} cells (n=12),

25 75±14% or Lin⁻c-kit^{NEG} cells (n=11), 75±15%.

To establish whether Lin⁻c-kit^{POS} cells resulted in myocardial regeneration, BrdU (50 mg/kg body weight, i.p.) was administered daily to the animals for 4-5 consecutive days before sacrifice to determine cumulative cell division during active growth. Sections were incubated with anti-BrdU antibody and BrdU labeling of

30 cardiac cell nuclei in the S phase was measured. Moreover, expression of Ki67 in nuclei (Ki67 is expressed in cycling cells in G1, S, G2, and early mitosis) was evaluated by treating samples with a rabbit polyclonal anti-mouse Ki67 antibody (Dako Corp.). FITC-conjugated goat anti-rabbit IgG was used as secondary

antibody. (Figure 5 and 6). EGFP was detected with a rabbit polyclonal anti-GFP (Molecular Probes). Myocytes were recognized with a mouse monoclonal anti-cardiac myosin heavy chain (MAB 1548; Chemicon) or a mouse monoclonal anti- α -sarcomeric actin (clone 5C5; Sigma), endothelial cells with a rabbit polyclonal anti-human factor VIII (Sigma) and smooth muscle cells with a mouse monoclonal anti- α -smooth muscle actin (clone 1A4; Sigma). Nuclei were stained with propidium iodide (PI), 10 μ g/ml. The percentages of myocyte (M), endothelial cell (EC) and smooth muscle cell (SMC) nuclei labeled by BrdU and Ki67 were obtained by confocal microscopy. This was accomplished by dividing the number of nuclei labeled by the total number of nuclei examined. Number of nuclei sampled in each cell population was as follows; BrdU labeling: M=2,908; EC=2,153; SMC=4,877. Ki67 labeling: M=3,771; EC=4,051; SMC=4,752. Number of cells counted for EGFP labeling: M=3,278; EC=2,056; SMC=1,274. The percentage of myocytes in the regenerating myocardium was determined by delineating the area occupied by cardiac myosin stained cells divided by the total area represented by the infarcted region in each case. Myocyte proliferation was 93% ($p<0.001$) and 60% ($p<0.001$) higher than in endothelial cells, and 225% ($p<0.001$) and 176% ($p<0.001$) higher than smooth muscle cells, when measured by BrdU and Ki67, respectively.

The origin of the cells in the forming myocardium was determined by the expression of EGFP (Fig. 7 and 8). EGFP expression was restricted to the cytoplasm and the Y chromosome to nuclei of new cardiac cells. EGFP was combined with labeling of proteins specific for myocytes, endothelial cells and smooth muscle cells. This allowed the identification of each cardiac cell type and the recognition of endothelial cells and smooth muscle cells organized in coronary vessels (Figs. 5, 7, and 8). The percentage of new myocytes, endothelial cells and smooth muscle cells that expressed EGFP was $53\pm 9\%$ ($n=7$), $44\pm 6\%$ ($n=7$) and $49\pm 7\%$ ($n=7$), respectively. These values were consistent with the fraction of transplanted $\text{Lin}^- \text{c-kit}^{\text{POS}}$ bone marrow cells that expressed EGFP, $44\pm 10\%$ ($n=6$). An average $54\pm 8\%$ ($n=6$) of myocytes, endothelial cells and smooth muscle cells expressed EGFP in the heart of donor transgenic mice.

E. Detection of the Y-Chromosome

For the fluorescence in situ hybridization (FISH) assay, sections were exposed to a denaturing solution containing 70% formamide. After dehydration

with ethanol, sections were hybridized with the DNA probe CEP Y (satellite III) Spectrum Green (Vysis) for 3 hours. Nuclei were stained with PI.

Y-chromosomes were not detected in cells from the surviving portion of the ventricle. However, the Y-chromosome was detected in the newly formed myocytes, indicating their origin as from the injected bone marrow cells (Fig. 9).

F. Detection of Transcription Factors and Connexin 43

Sections were incubated with rabbit polyclonal anti-MEF2 (C-21; Santa Cruz), rabbit polyclonal anti-GATA-4 (H-112; Santa Cruz), rabbit polyclonal anti-Csx/Nkx2.5 (obtained from Dr. Izumo) and rabbit polyclonal anti-connexin 43 (Sigma). FITC-conjugated goat anti-rabbit IgG (Sigma) was used as secondary antibody.

To confirm that newly formed myocytes represented maturing cells aiming at functional competence, the expression of the myocyte enhancer factor 2 (MEF2), the cardiac specific transcription factor GATA-4 and the early marker of myocyte development Csx/Nkx2.5 was examined. In the heart, MEF2 proteins are recruited by GATA-4 to synergistically activate the promoters of several cardiac genes such as myosin light chain, troponin T, troponin I, α -myosin heavy chain, desmin, atrial natriuretic factor and α -actin (Durocher et al., 1997; Morin et al., 2000).

Csx/Nkx2.5 is a transcription factor restricted to the initial phases of myocyte differentiation (Durocher et al., 1997). In the reconstituting heart, all nuclei of cardiac myosin labeled cells expressed MEF2 (Figs. 7D-7F) and GATA-4 (Fig. 10), but only 40 \pm 9% expressed Csx/Nkx2.5 (Figs. 7G-7I). To characterize further the properties of these myocytes, the expression of connexin 43 was determined. This protein is responsible for intercellular connections and electrical coupling through the generation of plasma membrane channels between myocytes (Beardsle et al., 1998; Musil et al., 2000); connexin 43 was apparent in the cell cytoplasm and at the surface of closely aligned differentiating cells (Figs. 11A-11D). These results were consistent with the expected functional competence of the heart muscle phenotype. Additionally, myocytes at various stages of maturation were detected within the same and different bands (Fig. 12).

EXAMPLE 2: Mobilization of Bone Marrow Cells to Repair Infarcted Myocardium

A. Myocardial Infarction and Cytokines.

Fifteen C57BL/6 male mice at 2 months of age were splenectomized and 2 weeks later were injected subcutaneously with recombinant rat stem cell factor (SCF), 200 µg/kg/day, and recombinant human granulocyte colony stimulating factor (G-CSF), 50 µg/kg/day (Amgen), once a day for 5 days (Bodine et al., 1994; Orlic et al., 1993). Under ether anesthesia, the left ventricle (LV) was exposed and the coronary artery was ligated (Orlic et al., 2001; Li et al., 1997; Li et al., 1999). SCF and G-CSF were given for 3 more days. Controls consisted of splenectomized infarcted and sham-operated (SO) mice injected with saline. BrdU, 50 mg/kg body weight, was given once a day, for 13 days, before sacrifice; mice were killed at 27 days. Protocols were approved by New York Medical College. Results are mean±SD. Significance was determined by the Student's *t* test and Bonferroni method (Li et al., 1999). Mortality was computed with log-rank test. *P*<0.05 was significant.

Given the ability of bone marrow Lin⁻ c-kit^{POS} cells to transdifferentiate into the cardiogenic lineage (Orlic et al., 2001), a protocol was used to maximize their number in the peripheral circulation in order to increase the probability of their homing to the region of dead myocardium. In normal animals, the frequency of Lin⁻ c-kit^{POS} cells in the blood is only a small fraction of similar cells present in the bone marrow (Bodine et al., 1994; Orlic et al., 1993). As documented previously, the cytokine treatment used here promotes a marked increase of Lin⁻ c-kit^{POS} cells in the bone marrow and a redistribution of these cells from the bone marrow to the peripheral blood. This protocol leads to a 250-fold increase in Lin⁻ c-kit^{POS} cells in the circulation (Bodine et al., 1994; Orlic et al., 1993).

In the current study, BMC mobilization by SCF and G-CSF resulted in a dramatic increase in survival of infarcted mice; with cytokine treatment, 73% of mice (11 of 15) survived 27 days, while mortality was very high in untreated infarcted mice (Fig. 13A). A large number of animals in this group died from 3 to 6 days after myocardial infarction (MI) and only 17% (9 of 52) reached 27 days (*p*<0.001). Mice that died within 48 hours post-MI were not included in the mortality curve to minimize the influence of the surgical trauma. Infarct size was similar in the cytokine-, 64±11% (*n*=11), and saline-, 62±9% (*n*=9), injected animals as measured by the number of myocytes lost in the left ventricular free wall (LVFW) at 27 days (Fig. 14).

Importantly, bone marrow cell mobilization promoted myocardial regeneration in all 11 cytokine-treated infarcted mice, sacrificed 27 days after surgery (Fig. 13B). Myocardial growth within the infarct was also seen in the 4 mice that died prematurely at day 6 (n=2) and at day 9 (n=2). Cardiac repair was characterized by a band of newly formed myocardium occupying most of the damaged area. The developing tissue extended from the border zone to the inside of the injured region and from the endocardium to the epicardium of the LVFW. In the absence of cytokines, myocardial replacement was never observed and healing with scar formation was apparent (Fig. 13C). Conversely, only small areas of collagen accumulation were detected in treated mice.

B. Detection of BMC Mobilization by Echocardiography and Hemodynamics.

Echocardiography was performed in conscious mice using a Sequoia 256c (Acuson) equipped with a 13-MHz linear transducer (15L8). The anterior chest area was shaved and two dimensional (2D) images and M-mode tracings were recorded from the parasternal short axis view at the level of papillary muscles. From M-mode tracings, anatomical parameters in diastole and systole were obtained (Pollick et al., 1995). Ejection fraction (EF) was derived from LV cross sectional area in 2D short axis view (Pollick et al., 1995): $EF = [(LVDA - LVSA) / LVDA] * 100$ where LVDA and LVSA correspond to LV areas in diastole and in systole. Mice were anesthetized with chloral hydrate (400 mg/kg body weight, ip) and a microtip pressure transducer (SPR-671, Millar) connected to a chart recorder was advanced into the LV for the evaluation of pressures and + and - dP/dt in the closed-chest preparation (Orlic et al., 2001; Li et al., 1997; Li et al., 1999).

EF was 48%, 62% and 114% higher in treated than in non-treated mice at 9, 16 and 26 days after coronary occlusion, respectively (Fig. 15D). In mice exposed to cytokines, contractile function developed with time in the infarcted region of the wall (Figs. 15E-M; Figs. 16H-P, www.pnas.org). Conversely, LV end-diastolic pressure (LVEDP) increased 76% more in non-treated mice. The changes in LV systolic pressure (not shown), developed pressure (LVDP), + and -dP/dt were also more severe in the absence of cytokine treatment (Figs. 17A-D). Additionally, the increase in diastolic stress in the zone bordering and remote from infarction was 69-73% lower in cytokine-treated mice (Fig. 15N). Therefore, cytokine-mediated

infarct repair restored a noticeable level of contraction in the regenerating myocardium, decreasing diastolic wall stress and increasing ventricular performance. Myocardial regeneration attenuated cavitory dilation and mural thinning during the evolution of the infarcted heart *in vivo*.

5 Echocardiographically, LV end-systolic (LVESD) and end-diastolic (LVEDD) diameters increased more in non-treated than in cytokine-treated mice, at 9, 16 and 26 days after infarction (Figs. 16A-B). Infarction prevented the evaluation of systolic (AWST) and diastolic (AWDT) anterior wall thickness. When measurable, the posterior wall thickness in systole (PWST) and diastole (PWDT)
10 was greater in treated mice (Figs. 16C-D). Anatomically, the wall bordering and remote from infarction was 26% and 22% thicker in cytokine-injected mice (Fig. 16E). BMC-induced repair resulted in a 42% higher wall thickness-to-chamber radius ratio (Fig. 15A). Additionally, tissue regeneration decreased the expansion in cavitory diameter, -14%, longitudinal axis, -5% (Figs. 16F-G), and chamber volume, -
15 26% (Fig. 15B). Importantly, ventricular mass-to-chamber volume ratio was 36% higher in treated animals (Fig. 15C). Therefore, BMC mobilization that led to proliferation and differentiation of a new population of myocytes and vascular structures attenuated the anatomical variables which define cardiac decompensation.

C. Cardiac Anatomy and Determination of Infarct Size.

20 Following hemodynamic measurements, the abdominal aorta was cannulated, the heart was arrested in diastole with CdCl_2 and the myocardium was perfused with 10% formalin. The LV chamber was filled with fixative at a pressure equal to the *in vivo* measured end-diastolic pressure (Li et al., 1997; Li et al., 1999). The LV intracavitory axis was measured and three transverse slices from the base,
25 mid-region and apex were embedded in paraffin. The mid-section was used to measure LV thickness, chamber diameter and volume (Li et al., 1997; Li et al., 1999). Infarct size was determined by the number of myocytes lost from the LVFW (Olivetti et al., 1991; Beltrami et al., 1994).

To quantify the contribution of the developing band to the ventricular mass,
30 firstly the volume of the LVFW (weight divided by 1.06 g/ml) was determined in each group of mice. The data was $56 \pm 2 \text{ mm}^3$ in sham operated (SO), $62 \pm 4 \text{ mm}^3$ (viable FW= 41 ± 3 ; infarcted FW= 21 ± 4) in infarcted non-treated animals, and $56 \pm 9 \text{ mm}^3$ (viable FW= 37 ± 8 ; infarcted FW= 19 ± 5) in infarcted cytokine-treated mice.

These values were compared to the expected values of spared and lost myocardium at 27 days, given the size of the infarct in the non-treated and cytokine-treated animals. From the volume of the LVFW (56 mm^3) in SO and infarct size in non-treated, 62%, and treated, 64%, mice, it was possible to calculate the volume of myocardium destined to remain (non-treated= 21 mm^3 ; treated= 20 mm^3) and destined to be lost (non-treated= 35 mm^3 ; treated= 36 mm^3) 27 days after coronary occlusion (Fig. 18A). The volume of newly formed myocardium was detected exclusively in cytokine-treated mice and found to be 14 mm^3 (Fig. 18A). Thus, the repair band reduced infarct size from 64% ($36 \text{ mm}^3 / 56 \text{ mm}^3 = 64\%$) to 39% [$(36 \text{ mm}^3 - 14 \text{ mm}^3) / 56 \text{ mm}^3 = 39\%$]. Since the spared portion of the LVFW at 27 days was 41 and 37 mm^3 in non-treated and treated mice (see above), the remaining myocardium, shown in Fig. 18a, underwent 95% ($p < 0.001$) and 85% ($p < 0.001$) hypertrophy, respectively. Consistently, myocyte cell volume increased 94% and 77% (Fig. 18B).

15 D. Determination the Total Volume of Formed Myocardium

The volume of regenerating myocardium was determined by measuring in each of three sections the area occupied by the restored tissue and section thickness. The product of these two variables yielded the volume of tissue repair in each section. Values in the three sections were added and the total volume of formed myocardium was obtained. Additionally, the volume of 400 myocytes was measured in each heart. Sections were stained with desmin and laminin antibodies and propidium iodide (PI). Only longitudinally oriented cells with centrally located nuclei were included. The length and diameter across the nucleus were collected in each myocyte to compute cell volume, assuming a cylindrical shape (Olivetti et al., 1991; Beltrami et al., 1994). Myocytes were divided in classes and the number of myocytes in each class was calculated from the quotient of total myocyte class volume and average cell volume (Kajstura et al., 1995; Reiss et al., 1996). Number of arteriole and capillary profiles per unit area of myocardium was measured as previously done (Olivetti et al., 1991; Beltrami et al., 1994).

30 Sections were incubated with BrdU or Ki67 antibody. Myocytes (M) were recognized with a mouse monoclonal anti-cardiac myosin, endothelial cells (EC) with a rabbit polyclonal anti-factor VIII and smooth muscle cells (SMC) with a mouse monoclonal anti- α -smooth muscle actin myosin. The fractions of M, EC and

SMC nuclei labeled by BrdU and Ki67 were obtained by confocal microscopy (Orlic et al., 2001). Nuclei sampled in 11 cytokine-treated mice; BrdU: M=3,541; EC=2,604; SMC=1,824. Ki67: M=3,096; EC=2,465; SMC=1,404.

BrdU was injected daily between days 14 to 26 to measure the cumulative extent of cell proliferation while Ki67 was assayed to determine the number of cycling cells at sacrifice. Ki67 identifies cells in G1, S, G2, prophase and metaphase, decreasing in anaphase and telophase (Orlic et al., 2001). The percentages of BrdU and Ki67 positive myocytes were 1.6- and 1.4-fold higher than EC, and 2.8- and 2.2-fold higher than SMC, respectively (Fig. 18C, 19). The forming myocardium occupied 76±11% of the infarct; myocytes constituted 61±12%, new vessels 12±5% and other components 3±2%. The band contained 15x10⁶ regenerating myocytes that were in an active growing phase and had a wide size distribution (Figs. 18D-E). EC and SMC growth resulted in the formation of 15±5 arterioles and 348±82 capillaries per mm² of new myocardium. Thick wall arterioles with several layers of SMC and luminal diameters of 10-30 µm represented vessels in early differentiation. At times, incomplete perfusion of the coronary branches within the repairing myocardium during the fixation procedure led to arterioles and capillaries containing erythrocytes (Figs. 18F-H). These results provided evidence that the new vessels were functionally competent and connected with the coronary circulation. Therefore, tissue repair reduced infarct size and myocyte growth exceeded angiogenesis; muscle mass replacement was the prevailing feature of the infarcted heart.

E. Determination of Cell Differentiation

Cytoplasmic and nuclear markers were used. Myocyte nuclei: rabbit polyclonal Csx/Nkx2.5, MEF2, and GATA4 antibodies (Orlic et al., 2001; Lin et al., 1997; Kasahara et al., 1998); cytoplasm: mouse monoclonal nestin (Kachinsky et al., 1995), rabbit polyclonal desmin (Hermann and Aebi, 1998), cardiac myosin, mouse monoclonal α-sarcomeric actin and rabbit polyclonal connexin 43 antibodies (Orlic et al., 2001). EC cytoplasm: mouse monoclonal flk-1, VE-cadherin and factor VIII antibodies (Orlic et al., 2001; Yamaguchi et al., 1993; Breier et al., 1996). SMC cytoplasm: flk-1 and α-smooth muscle actin antibodies (Orlic et al., 2001; Couper et al., 1997). Scar was detected by a mixture of collagen type I and type III antibodies.

Five cytoplasmic proteins were identified to establish the state of differentiation of myocytes (Orlic et al., 2001; Kachinsky et al., 1995; Hermann and Aebi, 1998): nestin, desmin, α -sarcomeric actin, cardiac myosin and connexin 43. Nestin was recognized in individual cells scattered across the forming band (Fig. 20A). With this exception, all other myocytes expressed desmin (Fig. 20B), α -sarcomeric actin, cardiac myosin and connexin 43 (Fig. 20C). Three transcription factors implicated in the activation of the promoter of several cardiac muscle structural genes were examined (Orlic et al., 2001; Lin et al., 1997; Kasahara et al., 1998): Csx/Nkx2.5, GATA-4 and MEF2 (Figs. 21A-C). Single cells positive for flk-1 and VE-cadherin (Yamaguchi et al., 1993; Breier et al., 1996), two EC markers, were present in the repairing tissue (Figs. 20D,E); flk-1 was detected in SMC isolated or within the arteriolar wall (Fig. 20F). This tyrosine kinase receptor promotes migration of SMC during angiogenesis (Couper et al., 1997). Therefore, repair of the infarcted heart involved growth and differentiation of all cardiac cell populations resulting in *de novo* myocardium.

EXAMPLE 3: Migration of Primitive Cardiac Cells in the Adult Mouse Heart

To determine whether a population of primitive cells was present in the adult ventricular myocardium and whether these cells possessed the ability to migrate, three major growth factors were utilized as chemoattractants: hepatocyte growth factor (HGF), stem cell factor (SCF) and granulocyte monocyte colony stimulating factor (GM-CSF). SCF and GMCSF were selected because they have been shown to promote translocation of hematopoietic stem cells. Although HGF induces migration of hematopoietic stem cells, this growth factor is largely implicated in mitosis, differentiation and migration of cardiac cell precursors during early cardiogenesis. On this basis, enzymatically dissociated cells from the mouse heart were separated according to their size. Methods for dissociating cardiac cells from heart tissue are well-known to those skilled in the art and therefore would not involve undue experimentation (*Cf* U.S. Patent No. 6,255,292 which is herein incorporated by reference in its entirety). A homogenous population of the dissociated cardiac cells containing small undifferentiated cells, 5-7 μ m in diameter, with a high nucleus to cytoplasm ratio were subjected to migration assay in Boyden

microchambers characterized by gelatin-coated filters containing pores, 5 μ m (Boyden et al., 1962, J. Exptl. Med. 115:453-456)

No major differences in the dose-response curve of migrated cells in the presence of the three growth factors were detected. However, HGF appeared to mobilize a larger number of cells at a concentration of 100 ng/ml. In addition, the cells that showed a chemotactic response to HGF consisted of 15% of *c-kit* positive (*c-kit*^{POS}) cells, 50% of multidrug resistance -1 (MDR-1) labeled cells and 30% of stem cell antigen-1 (Sca-1) expressing cells. When the mobilized cells were cultured in 15% fetal bovine serum, they differentiated into myocytes, endothelial cells, smooth muscle cells and fibroblasts. Cardiac myosin positive myocytes constituted 50% of the preparation, while factor VIII labeled cells included 15%, alpha-smooth muscle actin stained cells 4%, and vimentin positive factor VIII negative fibroblasts 20%. The remaining cells were small undifferentiated and did not stain with these four antibodies. In conclusion, the mouse heart possesses primitive cells which are mobilized by growth factors. HGF translocates cells that in vitro differentiate into the four cardiac cell lineages.

EXAMPLE 4: Cardiac *C-Kit* Positive Cells Proliferate *In Vitro* and Generate New Myocardium *Vivo*

To determine whether primitive *c-kit*^{POS} cells were present in senescent Fischer 344 rats, dissociated cardiac cells were exposed to magnetic beads coated with *c-kit* receptor antibody (ACK-4-biotin, anti-*c-kit* mAb). Following separation, these small undifferentiated cells were cultured in 10% fetal calf serum. Cells attached in a few days and began to proliferate at one week. Confluence was reached at 7-10 days. Doubling time, established at passage P2 and P4, required 30 and 40 hours, respectively. Cells grew up to P18 (90th generation) without reaching senescence. Replicative capacity was established by Ki67 labeling: at P2, 88 \pm 14% of the cells contained Ki67 protein in nuclei. Additional measurements were obtained between P1 and P4; 40% of cells expressed alpha-sarcomeric actin or cardiac myosin, 13% desmin, 3% alpha-smooth muscle actin, 15% factor VIII or CD31, and 18% nestin. Under these *in vitro* conditions, cells showed no clear myofibrillar organization with properly aligned sarcomeres and spontaneous contraction was never observed. Similarly, Ang II, norepinephrine, isoproterenol,

mechanical stretch and electrical field stimulation failed to initiate contractile function. On this basis, it was decided to evaluate whether these cells pertaining to the myogenic, smooth muscle cell and endothelial cell lineages had lost permanently their biological properties or their role could be reestablished *in vivo*. Following
5 BrdU labeling of cells at P2, infarcted Fischer 344 rats were injected with these BrdU positive cells in the damaged region, 3-5 hours after coronary artery occlusion. Two weeks later, animals were sacrificed and the characteristics of the infarcted area were examined. Myocytes containing parallel arranged myofibrils along their longitudinal axis were recognized, in combination with BrdU labeling of nuclei.
10 Moreover, vascular structures comprising arterioles and capillary profiles were present and were also positive to BrdU. In conclusion, primitive *c-kit* positive cells reside in the senescent heart and maintain the ability to proliferate and differentiate into parenchymal cells and coronary vessels when implanted into injured functionally depressed myocardium.

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EXAMPLE 5: Cardiac Stem Cells Mediate Myocyte Replication in the Young and Senescent Rat Heart

The heart is not a post-mitotic organ but contains a subpopulation of myocytes that physiologically undergo cell division to replace dying cells. Myocyte
20 multiplication is enhanced during pathologic overloads to expand the muscle mass and maintain cardiac performance. However, the origin of these replicating myocytes remains to be identified. Therefore, primitive cells with characteristics of stem/progenitor cells were searched for in the myocardium of Fischer 344 rats. Young and old animals were studied to determine whether aging had an impact on
25 the size population of stem cells and dividing myocytes. The numbers of *c-kit* and MDR1 positive cells in rats at 4 months were 11 ± 3 , and $18 \pm 6/100 \text{ mm}^2$ of tissue, respectively. Values in rats at 27 months were 35 ± 10 , and $42 \pm 13/100 \text{ mm}^2$. A number of newly generated small myocytes were identified that were still *c-kit* or MDR1 positive. Ki67 protein, which is expressed in nuclei of cycling cells was
30 detected in $1.3 \pm 0.3\%$ and $4.1 \pm 1.5\%$ of myocytes at 4 and 27 months, respectively. BrdU localization following 6 or 56 injections included $1.0 \pm 0.4\%$ and $4.4 \pm 1.2\%$ at 4 months, and $4.0 \pm 1.5\%$ and $16 \pm 4\%$ at 27 months. The mitotic index measured in tissue sections showed that the fraction of myocyte nuclei in mitosis comprised

82±28/10⁶ and 485±98/10⁶ at 4 and 27 months, respectively. These determinations were confirmed in dissociated myocytes to obtain a cellular mitotic index. By this approach, it was possible to establish that all nuclei of multinucleated myocytes were in mitosis simultaneously. This information could not be obtained in tissue sections. The collected values showed that 95±31 /10⁶ myocytes were dividing at 4 months and 620±98/10⁶ at 27 months. At both age intervals, the formation of the mitotic spindle, contractile ring, disassembly of the nuclear envelope, karyokinesis and cytokinesis were documented. In conclusion, primitive undifferentiated cells reside in the adult heart and their increase with age is paralleled by an increase in the number of myocytes entering the cell cycle and undergoing karyokinesis and cytokinesis. This relationship suggests that cardiac stem cells may regulate the level and fate of myocyte growth in the aging heart.

EXAMPLE 6: Chimerism of the Human Heart and the Role of stem Cells

The critical role played by resident primitive cells in the remodeling of the injured heart is well appreciated when organ chimerism, associated with transplantation of a female heart in a male recipient, is considered. For this purpose, 8 female hearts implanted in male hosts were analyzed. Translocation of male cells to the grafted female heart was identified by FISH for Y chromosome (see Example 1E). By this approach, the percentages of myocytes, coronary arterioles and capillary profiles labeled by Y chromosome were 9%, 14% and 7%, respectively. Concurrently, the numbers of undifferentiated *c-kit* and multidrug resistance-1 (MDR1) positive cells in the implanted female hearts were measured. Additionally, the possibility that these cells contained the Y chromosome was established. Cardiac transplantation involves the preservation of portions of the atria of the recipient on which the donor heart with part of its own atria is attached. This surgical procedure is critical for understanding whether the atria from the host and donor contained undifferentiated cells that may contribute to the complex remodeling process of the implanted heart. Quantitatively, the values of *c-kit* and MDR1 labeled cells were very low in control non-transplanted hearts: 3 *c-kit* and 5 MDR1/100 mm² of left ventricular myocardium. In contrast, the numbers of *c-kit* and MDR1 cells in the atria of the recipient were 15 and 42/100 mm².

Corresponding values in the atria of the donor were 15 and 52/100 mm² and in the ventricle 11 and 21/100 mm². Transplantation was characterized by a marked increase in primitive undifferentiated cells in the heart. Stem cells in the atria of the host contained Y chromosome, while an average of 55% and 63% of *c-kit* and MDR1 cells in the donor's atria and ventricle, respectively, expressed the Y chromosome. All *c-kit* and MDR1 positive cells were negative for CD45. These observations suggest that the translocation of male cells to the implanted heart has a major impact on the restructuring of the donor myocardium. In conclusion, stem cells are widely distributed in the adult heart and because of their plasticity and migration capacity generate myocytes, coronary arterioles and capillary structures with high degree of differentiation.

EXAMPLE 7: Identification and Localization of Stem Cells In The Adult Mouse Heart

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Turnover of myocytes occurs in the normal heart, and myocardial damage leads to activation of myocyte proliferation and vascular growth. These adaptations raise the possibility that multipotent primitive cells are present in the heart and are implicated in the physiological replacement of dying myocytes and in the cellular growth response following injury. On this basis, the presence of undifferentiated cells in the normal mouse heart was determined utilizing surface markers including *c-kit*, which is the receptor for stem cell factor, multidrug resistance-1 (MDR1), which is a P-glycoprotein capable of extruding from the cell dyes, toxic substances and drugs, and stem cell antigen-1 (Sca-1), which is involved in cell signaling and cell adhesion. Four separate regions consisting of the left and right atria, and the base, mid-section and apical portion of the ventricle were analyzed. From the higher to the lower value, the number of *c-kit* positive cells was 26±11, 15±5, 10±7 and 6±3/100 mm² in the atria, and apex, base and mid-section of the ventricle, respectively. In comparison with the base and mid-section, the larger fraction of *c-kit* positive cells in the atria and apex was statistically significant. The number of MDR1 positive cells was higher than those expressing *c-kit*, but followed a similar localization pattern; 43±14, 29±16, 14±7 and 12±10/100 mm² in the atria, apex, base and mid-section. Again, the values in the atria and apex were greater than in the

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other two areas. Sca-1 labeled cells showed the highest value; $150 \pm 36/100 \text{ mm}^2$ positive cells were found in the atria. Cells positive for *c-kit*, MDR1 and Sca-1 were negative for CD45, and for myocyte, endothelial cell, smooth muscle cell and fibroblast cytoplasmic proteins. Additionally, the number of cells positive to both c-kit and MDR1 was measured to recognize cells that possessed two stem cell markers. In the entire heart, 36% of *c-kit* labeled cells expressed MDR1 and 19% of MDR1 cells had also *c-kit*. In conclusion, stem cells are distributed throughout the mouse heart, but tend to accumulate in the regions at low stress, such as the atria and the apex.

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Having thus described in detail preferred embodiments of the present invention, it is to be understood that the invention defined by the appended claims is not to be limited by particular details set forth in the above description as many apparent variations thereof are possible without departing from the spirit or scope thereof.

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I CLAIM:

1. A method for repairing and/or generating and/or regenerating myocardium and/or myocardial cells comprising the administration of somatic stem cells.
5
2. The method of claim 1, wherein the administered somatic stem cells are adult stem cells.
- 10 3. The method of claim 1, wherein the administered somatic stem cells are cardiac stem cells.
4. The method of claim 1, wherein the administered somatic stem cells are adult cardiac stem cells.
15
5. The method of claim 1, wherein the administered somatic stem cells are cardiac stem cells and stem cells of another type other than cardiac stem cells.
- 20 6. The method of claim 1, wherein the administered somatic stem cells are adult cardiac stem cells and stem cells of another type other than adult cardiac stem cells.
7. The method of claim 1, wherein the administered somatic stem cells are
25 cardiac and hematopoietic stem cells.
8. The method of claim 1, wherein the administered somatic stem cells are adult cardiac and adult hematopoietic stem cells.
- 30 9. The method of claim 1, wherein the somatic stem cells are lineage negative.
10. The method of claim 9, wherein the lineage negative somatic stem cells are $c\text{-kit}^{\text{POS}}$.

11. The method of claim 1, wherein a therapeutically effective dose of somatic stem cells are delivered to the heart.
- 5 12. The method of claim 11, wherein the therapeutically effective dose of the somatic stem cells is $2 \times 10^4 - 1 \times 10^5$ cells.
13. The method of claim 11, wherein the therapeutically effective dose is delivered to the border area of the damaged myocardium.
- 10 14. The method of claim 11, wherein the therapeutically effective dose is administered by injection.
- 15 15. The method of claim 13, wherein the therapeutically effective dose is injected intramyocardially.
16. The method of claim 15, wherein the therapeutically effective dose is injected trans-epicardially or transendocardially.
- 20 17. The method of claim 16, wherein with the trans-endocardial approach a catheter-based approach is used.
18. The method of claim 13, wherein the administered somatic stem cells migrate into the damaged myocardium.
- 25 19. The method of claim 18, wherein the delivered somatic stem cells differentiate into one or more of the following types of cells selected from the group consisting of:
- a. myocytes;
 - 30 b. smooth muscle cells; and
 - c. endothelial cells.

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20. The method of claim 19, wherein the differentiated somatic stem cells proliferate.
21. The method of claim 19, wherein the differentiated somatic stem cells assemble into myocardium and/or myocardial cells.
22. The method of claim 19, wherein the differentiated somatic stem cells assemble into a coronary artery.
23. The method of claim 19, wherein the differentiated somatic stem cells assemble into an arteriole.
24. The method of claim 19, wherein the differentiated somatic stem cells assemble into a capillary.
25. The method of claim 19, wherein the differentiated somatic stem cells at least partially restore structural and functional integrity to the damaged myocardium and/or myocardial cells.
26. A method of repairing and/or generating and/or regenerating recently damaged myocardium and/or myocardial cells comprising the administration of a cytokine.
27. The method of claim 26, wherein the cytokine comprises a cytokine selected from the group consisting of:
- a. a stem cell factor;
 - b. a granulocyte-colony stimulating factor;
 - c. a stromal cell-derived factor-1;
 - d. an interleukin-3;
 - e. a granulocyte-macrophage colony stimulating factor;
 - f. a macrophage colony stimulating factor;
 - g. a steel factor;
 - h. a vascular endothelial growth factor;

and combinations thereof.

28. The method of claim 27, wherein a therapeutically effective dose of a cytokine is administered.
- 5 29. The method of claim 28, wherein the therapeutically effective dose of the cytokine is 50-500 µg/kg per day.
30. The method of claim 28, wherein the therapeutically effective dose is injected.
- 10 31. The method of claim 30, wherein the therapeutically effective dose is injected subcutaneously.
- 15 32. The method of claim 30, wherein the therapeutically effective dose is injected intravenously.
33. The method of claim 28, wherein the therapeutically effective dose stimulates the patient's own somatic stem cells.
- 20 34. The method of claim 33, wherein the stimulated somatic stem cells are adult stem cells.
35. The method of claim 33, wherein the stimulated somatic stem cells are hematopoietic stem cells.
- 25 36. The method of claim 33, wherein the stimulated somatic stem cells are adult hematopoietic stem cells.
- 30 37. The method of claim 33, wherein the stimulated somatic stem cells are cardiac stem cells.

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38. The method of claim 33, wherein the stimulated somatic stem cells are adult cardiac stem cells.
39. The method of claim 33, wherein the stimulated somatic stem cells are cardiac stem cells and stem cells of another type of stem cell other than cardiac stem cells.
40. The method of claim 33, wherein the stimulated somatic stem cells are adult cardiac stem cells and stem cells of another type of stem cell other than adult cardiac stem cells.
41. The method of claim 33, wherein the stimulated somatic stem cells are cardiac and hematopoietic stem cells.
42. The method of claim 33, wherein the stimulated somatic stem cells are adult cardiac and adult hematopoietic stem cells.
43. The method of claim 33, wherein the stimulated somatic stem cells become mobilized.
44. The method of claim 43, wherein the mobilized somatic stem cells home to the damaged myocardium and/or myocardial cells.
45. The method of claim 43, wherein the mobilized somatic stem cells migrate into the damaged myocardium and/or myocardial cells.
46. The method of claim 44, wherein the somatic stem cells differentiate into one or more of the following types of cells selected from the group consisting of:
- a. myocytes;
 - b. smooth muscle cells; and
 - c. endothelial cells.

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47. The method of claim 46, wherein the differentiated somatic stem cells proliferate.
48. The method of claim 46 wherein the differentiated somatic stem cells assemble into myocardium and/or myocardial cells.
49. The method of claim 46, wherein the differentiated somatic stem cells assemble into a coronary artery.
50. The method of claim 46, wherein the differentiated somatic stem cells assemble into an arteriole.
51. The method of claim 46, wherein the differentiated somatic stem cells assemble into a capillary.
52. The method of claim 46, wherein the differentiated somatic stem cells at least partially restore function to the damaged myocardium and/or myocardial cells.
53. A method of repairing and/or generating and/or regenerating recently damaged myocardium and/or myocardial cells comprising the administration of somatic stem cells and a cytokine.
54. The method of claim 53, wherein the administered somatic stem cells are adult stem cells.
55. The method of claim 53, wherein the administered somatic stem cells are hematopoietic stem cells.
56. The method of claim 53, wherein the administered somatic stem cells are adult hematopoietic stem cells.

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57. The method of claim 53, wherein the administered somatic stem cells are cardiac stem cells.
58. The method of claim 53, wherein the administered somatic stem cells are adult cardiac stem cells.
59. The method of claim 53, wherein the administered somatic stem cells are cardiac stem cells and stem cells of another type of stem cell other than cardiac stem cells.
60. The method of claim 53, wherein the administered somatic stem cells are adult cardiac stem cells and stem cells of another type of stem cell other than adult cardiac stem cells.
61. The method of claim 53, wherein the administered somatic stem cells are cardiac and hematopoietic stem cells.
62. The method of claim 53, wherein the administered somatic stem cells are adult cardiac and adult hematopoietic stem cells.
63. The method of claim 53, wherein the administered somatic stem cells are lineage negative.
64. The method of claim 63, wherein the administered lineage negative somatic stem cells are *c-kit*^{POS}.
65. The method of claim 53, wherein a therapeutically effective dose of somatic stem cells is delivered to the heart.
66. The method of claim 65, wherein the therapeutically effective dose of the somatic stem cells is $2 \times 10^4 - 1 \times 10^5$ cells.

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67. The method of claim 65, wherein the therapeutically effective dose is administered by injection.
68. The method of claim 67, wherein the therapeutically effective dose is injected mintramyocardially.
69. The method of claim 67, wherein the therapeutically effective dose is injected trans-epicardially or transendocardially.
70. The method of claim 69, wherein with the trans-endocardial approach a catheter-based approach is used.
71. The method of claim 53, where in the cytokine comprises a cytokine selected from the group consisting of:
- a. a stem cell factor;
 - b. a granulocyte-colony stimulating factor;
 - c. a stromal cell-derived factor-1;
 - d. an interleukin-3;
 - e. a granulocyte-macrophage colony stimulating factor;
 - f. a macrophage colony stimulating factor;
 - g. a steel factor;
 - h. a vascular endothelial growth factor;
- and combinations thereof.
72. The method of claim 71, wherein a therapeutically effective dose of a cytokine is administered.
73. The method of claim 72, wherein the therapeutically effective dose of the cytokine is 50-500 $\mu\text{g/kg}$ per day.
74. The method of claim 72, wherein the therapeutically effective dose is injected.

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75. The method of claim 74, wherein the therapeutically effective dose is injected subcutaneously.
76. The method of claim 74, wherein the therapeutically effective dose is injected intravenously.
77. The method of claim 74, wherein the therapeutically effective dose stimulates the patient's somatic stem cells.
78. The method of claim 77, wherein the stimulated somatic stem cells are mobilized.
79. The method of claim 77, wherein the stimulated somatic stem cells home to the damaged myocardium and/or myocardial cells.
80. The method of claim 79, wherein the somatic stem cells migrate into the damaged myocardium and/or myocardial cells.
81. The method of claim 80, wherein the somatic stem cells differentiate into one or more of the following types of cells selected from the group consisting of:
- a. myocytes;
 - b. smooth muscle cells; and
 - c. endothelial cells.
82. The method of claim 81, wherein the differentiated somatic stem cells proliferate.
83. The method of claim 82, wherein the differentiated somatic stem cells assemble into myocardium and/or myocardial cells.
84. The method of claim 82, wherein the differentiated somatic stem cells assemble into a coronary artery.

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85. The method of claim 82, wherein the differentiated somatic stem cells assemble into an arteriole.
86. The method of claim 82, wherein the differentiated somatic stem cells assemble into a capillary.
87. The method of claim 82, wherein the differentiated somatic stem cells at least partially restore structural and functional integrity to the damaged myocardium and/or myocardial cells.
88. A pharmaceutical composition comprising a therapeutically effective amount of somatic stem cells.
89. The pharmaceutical composition of claim 88, wherein the administered somatic stem cells are adult stem cells.
90. The pharmaceutical composition of claim 88, wherein the administered somatic stem cells are hematopoietic stem cells.
91. The pharmaceutical composition of claim 88, wherein the administered somatic stem cells are adult hematopoietic stem cells.
92. The pharmaceutical composition of claim 88, wherein the administered somatic stem cells are cardiac stem cells.
93. The pharmaceutical composition of claim 88, wherein the administered somatic stem cells are adult cardiac stem cells.
94. The pharmaceutical composition of claim 88, wherein the administered somatic stem cells are cardiac stem cells and stem cells of another type of stem cell other than cardiac stem cells.

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95. The pharmaceutical composition of claim 88, wherein the administered somatic stem cells are adult cardiac stem cells and stem cells of another type of stem cell other than adult cardiac stem cells.
- 5 96. The pharmaceutical composition of claim 88, wherein the administered somatic stem cells are cardiac and hematopoietic stem cells.
97. The pharmaceutical composition of claim 88, wherein the administered somatic stem cells are adult cardiac and adult hematopoietic stem cells.
- 10 98. The pharmaceutical composition of claim 88, wherein the therapeutically effective dose of the somatic stem cells is $2 \times 10^4 - 1 \times 10^5$ cells.
99. A pharmaceutical composition comprising a therapeutically effective amount
15 of somatic stem cells and a cytokine comprising a cytokine selected from the group consisting of:
- a. a stem cell factor;
 - b. a granulocyte-colony stimulating factor;
 - 20 c. a stromal cell-derived factor-1;
 - d. an interleukin-3;
 - e. a granulocyte-macrophage colony stimulating factor;
 - f. a macrophage colony stimulating factor;
 - g. a steel factor;
 - 25 h. vascular endothelial growth factor;
- and combinations thereof.
100. The pharmaceutical composition of claim 99 wherein the therapeutically effective dose of the cytokine is 50-500 µg/kg per day.
- 30 101. A composition comprising a therapeutically effective amount of somatic stem cells.

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102. The composition of claim 101, wherein the administered somatic stem cells are adult stem cells.
103. The composition of claim 101, wherein the administered somatic stem cells are hematopoietic stem cells.
104. The composition of claim 101, wherein the administered somatic stem cells are adult hematopoietic stem cells.
105. The composition of claim 101, wherein the administered somatic stem cells are cardiac stem cells.
106. The composition of claim 101, wherein the administered somatic stem cells are adult cardiac stem cells.
107. The composition of claim 101, wherein the administered somatic stem cells are cardiac stem cells and stem cells of another type of stem cell other than cardiac stem cells.
108. The composition of claim 101, wherein the administered somatic stem cells are adult cardiac stem cells and stem cells of another type of stem cell other than adult cardiac stem cells.
109. The composition of claim 101, wherein the administered somatic stem cells are cardiac and hematopoietic stem cells.
110. A method of implanting or depositing cells or causing the implantation or depositing of somatic stem cells in cardiac or blood vessel tissue comprising administration of somatic stem cells.
111. The method of claim 110, wherein the administered somatic stem cells are adult stem cells.

112. The method of claim 110, wherein the administered somatic stem cells are hematopoietic stem cells.
113. The method of claim 110, wherein the administered somatic stem cells are adult hematopoietic stem cells.
114. The method of claim 110, wherein the administered somatic stem cells are cardiac stem cells.
115. The method of claim 110, wherein the administered somatic stem cells are adult cardiac stem cells.
116. The method of claim 110, wherein the administered somatic stem cells are cardiac stem cells and stem cells of another type other than cardiac stem cells.
117. The method of claim 110, wherein the administered somatic stem cells are adult cardiac stem cells and stem cells of another type other than adult cardiac stem cells.
118. The method of claim 110, wherein the administered somatic stem cells are cardiac and hematopoietic stem cells.
119. The method of claim 110, wherein the administered somatic stem cells are adult cardiac and adult hematopoietic stem cells.
120. The method of claim 110, wherein the somatic stem cells are lineage negative.
121. The method of claim 120, wherein the lineage negative somatic stem cells are *c-kit*^{POS}.

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122. The method of claim 110, wherein a therapeutically effective dose of somatic stem cells are delivered to the heart.
123. The method of claim 110, wherein the therapeutically effective dose of the somatic stem cells is $2 \times 10^4 - 1 \times 10^5$ cells.
124. A method of implanting or depositing cells or causing the implantation or depositing of somatic stem cells in cardiac or blood vessel tissue comprising administration of a cytokine.
125. The method of claim 124, wherein the cytokine comprises a cytokine selected from the group consisting of:
- i. a stem cell factor;
 - j. a granulocyte-colony stimulating factor;
 - k. a stromal cell-derived factor-1;
 - l. an interleukin-3;
 - m. a granulocyte-macrophage colony stimulating factor;
 - n. a macrophage colony stimulating factor;
 - o. a steel factor;
 - p. a vascular endothelial growth factor;
- and combinations thereof.
126. The method of claim 124, wherein a therapeutically effective dose of a cytokine is administered.
127. The method of claim 126, wherein the therapeutically effective dose of the cytokine is 50-500 $\mu\text{g/kg}$ per day.
128. The method of claim 126, wherein the therapeutically effective dose stimulates the patient's own somatic stem cells.
129. The method of claim 128, wherein the stimulated somatic stem cells are adult stem cells.

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130. The method of claim 128, wherein the stimulated somatic stem cells are hematopoietic stem cells.
131. The method of claim 128, wherein the stimulated somatic stem cells are adult
5 hematopoietic stem cells.
132. The method of claim 128, wherein the stimulated somatic stem cells are cardiac stem cells.
- 10 133. The method of claim 128, wherein the stimulated somatic stem cells are adult cardiac stem cells.
134. The method of claim 128, wherein the stimulated somatic stem cells are cardiac stem cells and stem cells of another type of stem cell other than
15 cardiac stem cells.
135. The method of claim 128, wherein the stimulated somatic stem cells are adult cardiac stem cells and stem cells of another type of stem cell other than adult
20 cardiac stem cells.
136. The method of claim 128, wherein the stimulated somatic stem cells are cardiac and hematopoietic stem cells.
137. The method of claim 128, wherein the stimulated somatic stem cells are adult
25 cardiac and adult hematopoietic stem cells.
138. The method of claim 128, wherein the stimulated somatic stem cells become mobilized.
- 30 139. The method of claim 138, wherein the mobilized somatic stem cells home to the damaged myocardium and/or myocardial cells.

140. The method of claim 138, wherein the mobilized somatic stem cells migrate into the damaged myocardium and/or myocardial cells.
141. The composition of claim 101, wherein the administered somatic stem cells are adult cardiac and adult hematopoietic stem cells.
142. The composition of claim 101, wherein the therapeutically effective dose of the somatic stem cells is $2 \times 10^4 - 1 \times 10^5$ cells.
143. A composition comprising a therapeutically effective amount of somatic stem cells and a cytokine comprising a cytokine selected from the group of:
- i. a stem cell factor;
 - j. a granulocyte-colony stimulating factor;
 - k. a stromal cell-derived factor-1;
 - l. an interleukin-3;
 - m. a granulocyte-macrophage colony stimulating factor;
 - n. a macrophage colony stimulating factor;
 - o. a steel factor;
 - p. vascular endothelial growth factor;
- and combinations thereof.
144. The composition of claim 112 wherein the therapeutically effective dose of the cytokine is 50-500 $\mu\text{g/kg}$ per day.
145. A pharmaceutical composition for use in the treatment, therapy or prevention of cardiovascular disease or related complaint.
146. A pharmaceutical composition for use in repairing and/or generating and/or regenerating recently damaged myocardium and/or myocardial cells.

147. A kit comprising a pharmaceutical composition of claims 88 or 99 for use in the treatment, therapy or prevention of cardiovascular disease or related complaint.
- 5 148. A kit comprising a pharmaceutical composition of claims 88 or 99 for use in repairing and/or generating and/or regenerating recently damaged myocardium and/or myocardial cells.
- 10 149. A kit comprising somatic stem cells and optionally a cytokine for the treatment, therapy or prevention of cardiovascular disease or related complaint.
150. A kit for preparing pharmaceutical compositions of claims 88 or 99.
- 15 151. A method of making pharmaceutical compositions of claims 88 or 99.
152. A method of making compositions of claim 101 or 112.
- 20 153. A method of treating damaged myocardium and/or myocardial cells comprising the steps of:
- a. harvesting somatic stem cells;
 - b. *in vitro* culturing of the harvested somatic stem cells in conjunction with heart tissue, optionally in the presence of a cytokine;
 - 25 c. *in vitro* differentiation of the somatic stem cells into myocytes, smooth muscle cells and endothelial cells;
 - d. *in vitro* proliferation of differentiated cells and formation of tissue;
 - 30 e. *in vitro* assembly of differentiated cells and/or tissue into cardiac structures, including coronary arteries, arterioles, capillaries, and myocardium.
 - f. implanting the cultured somatic stem cells, tissue, or cardiac structures into the heart.

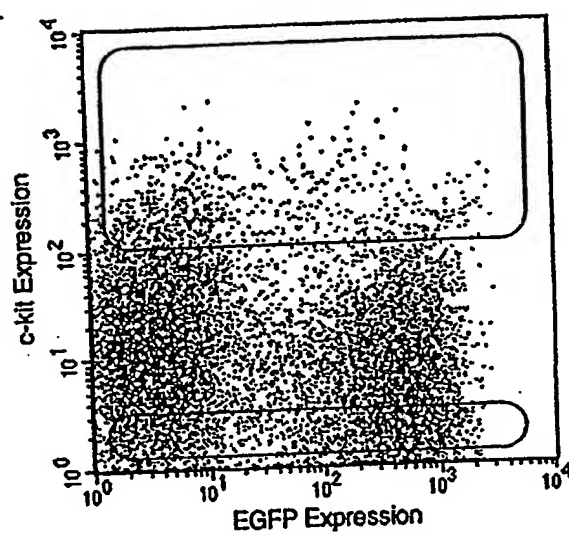


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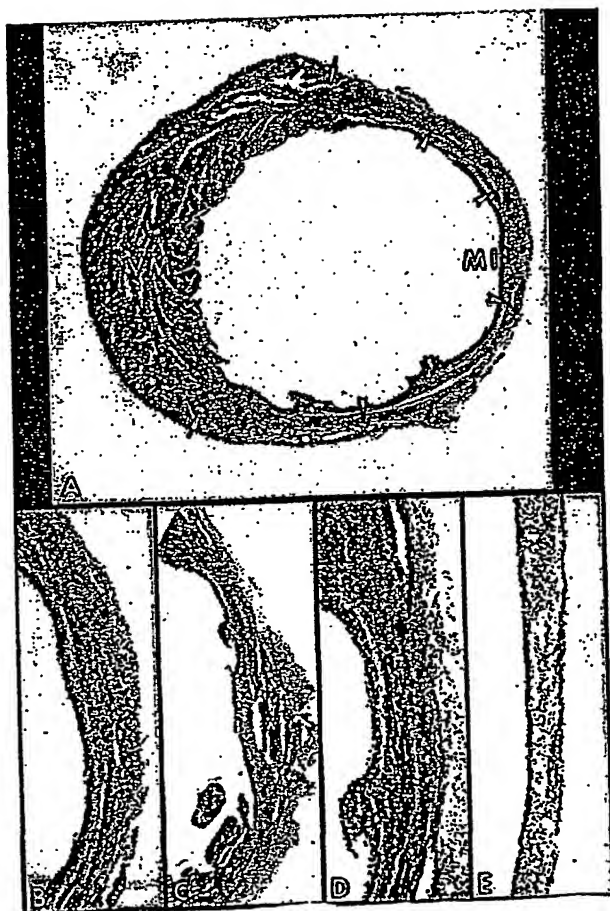


Figure 2

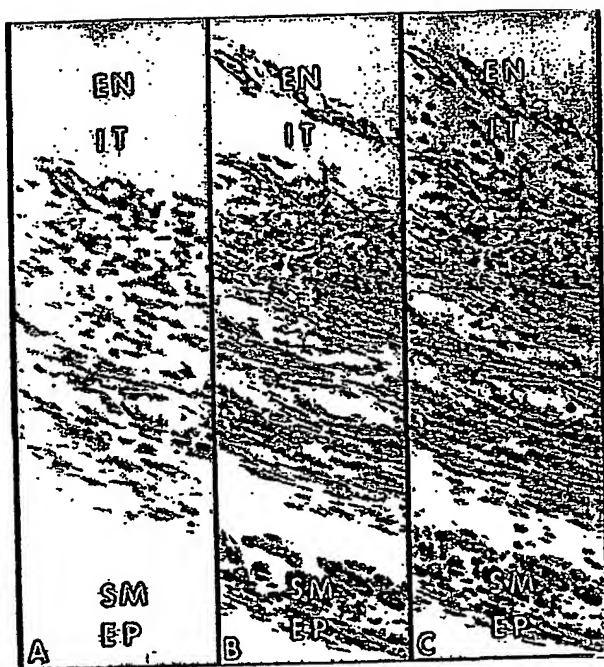


Figure 3.

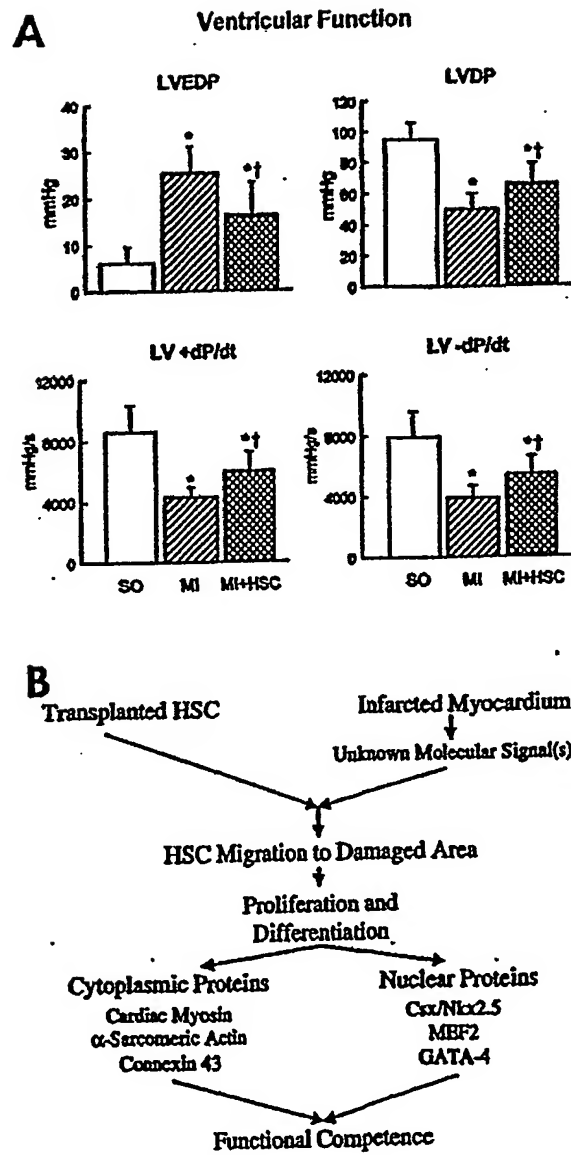


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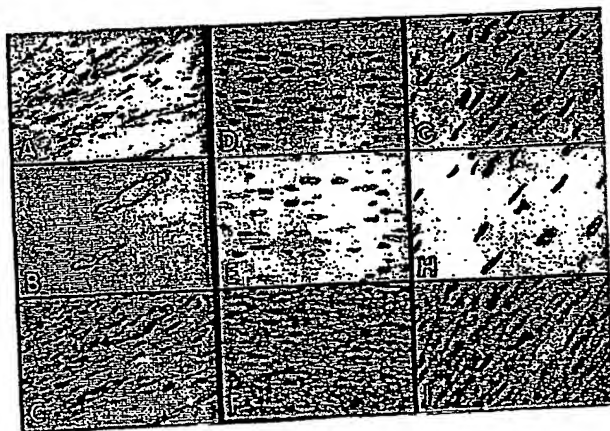


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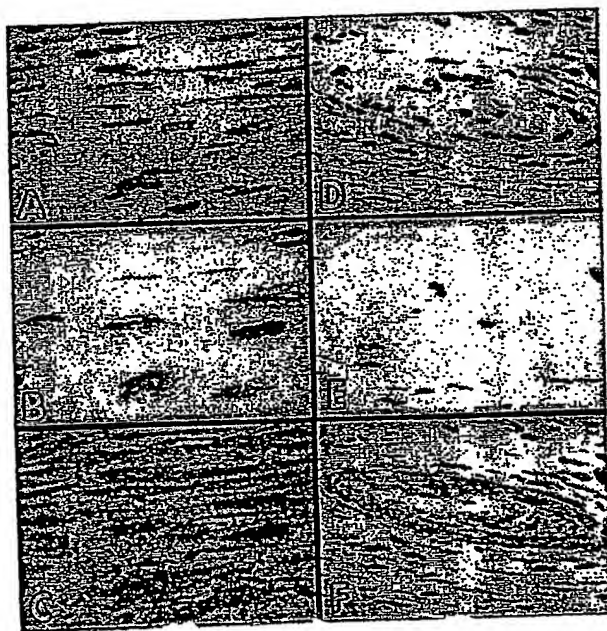


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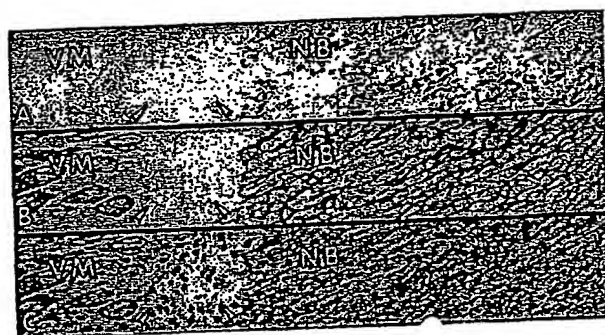


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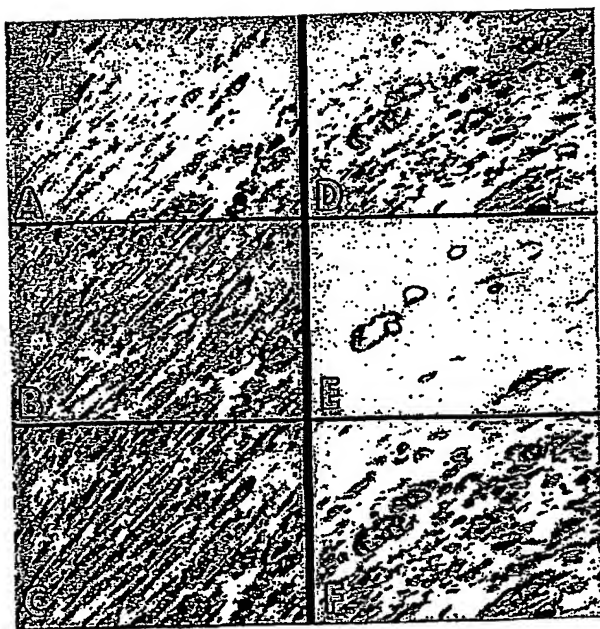


Figure 8

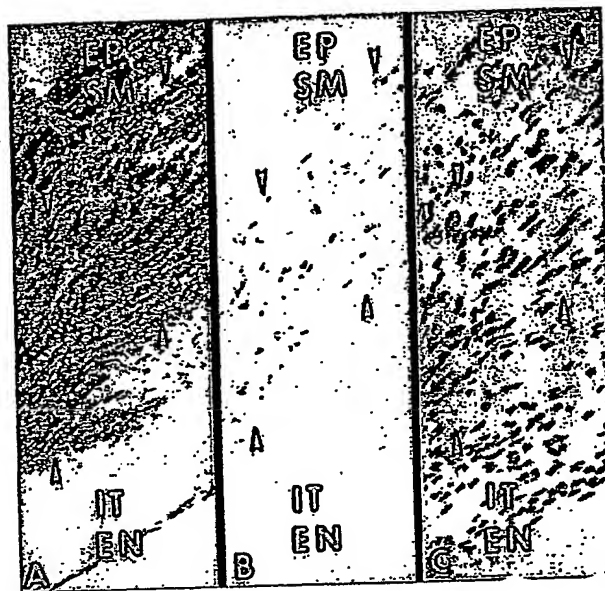


Figure 9

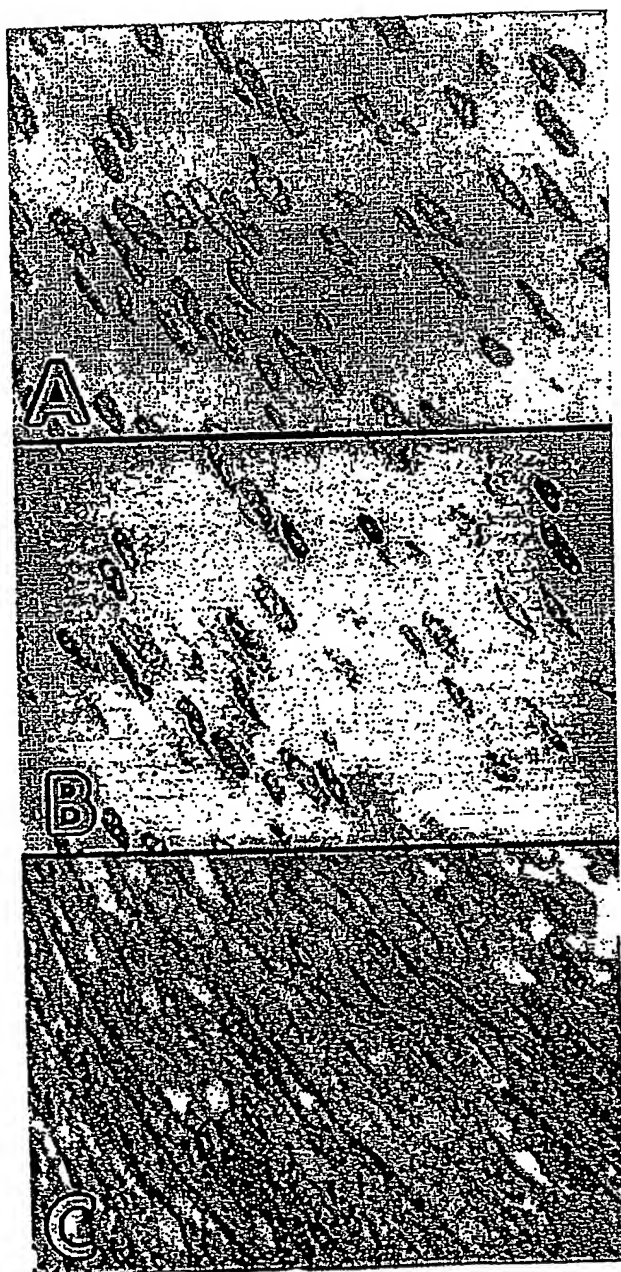


Figure 10



Figure 11



Figure 12

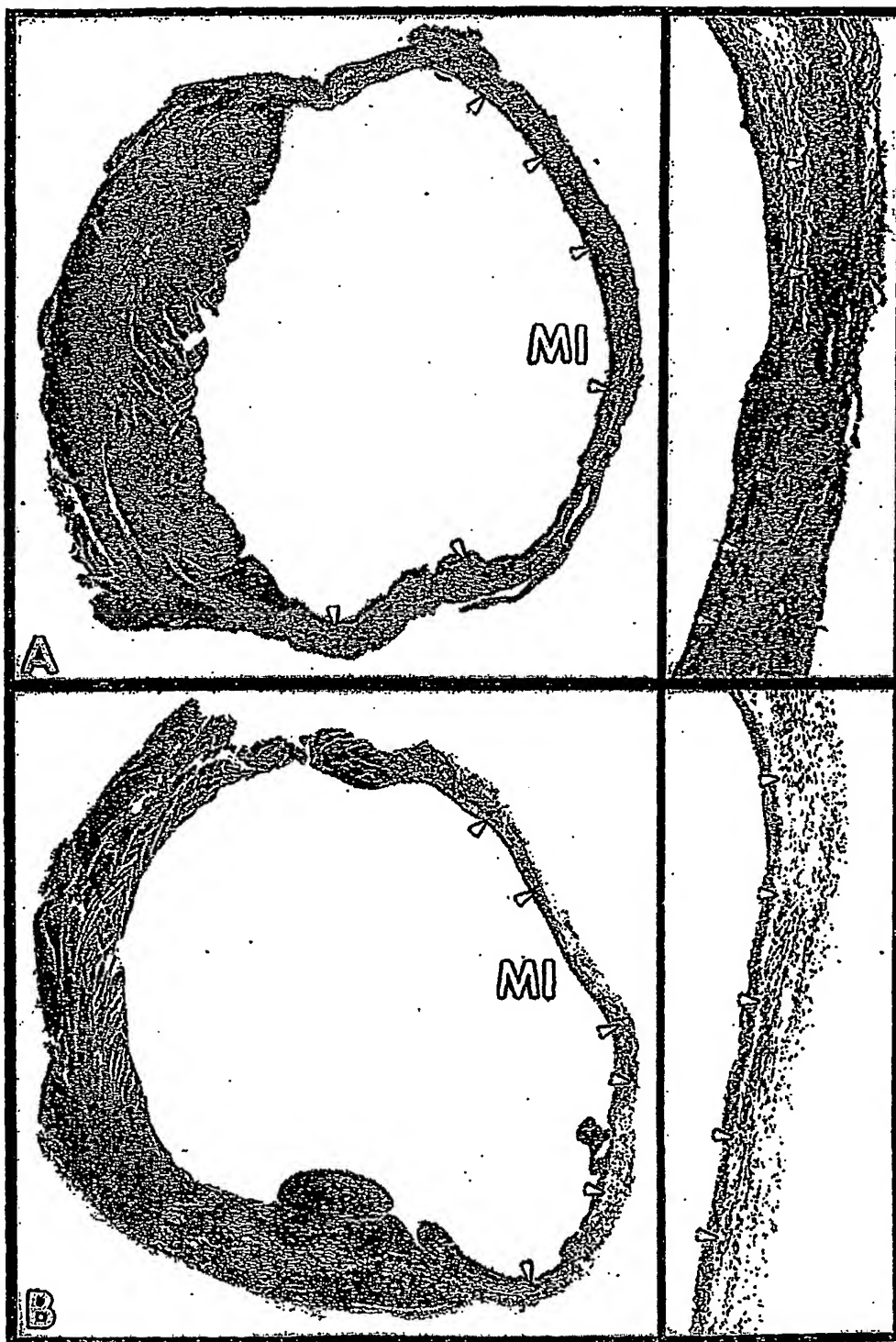


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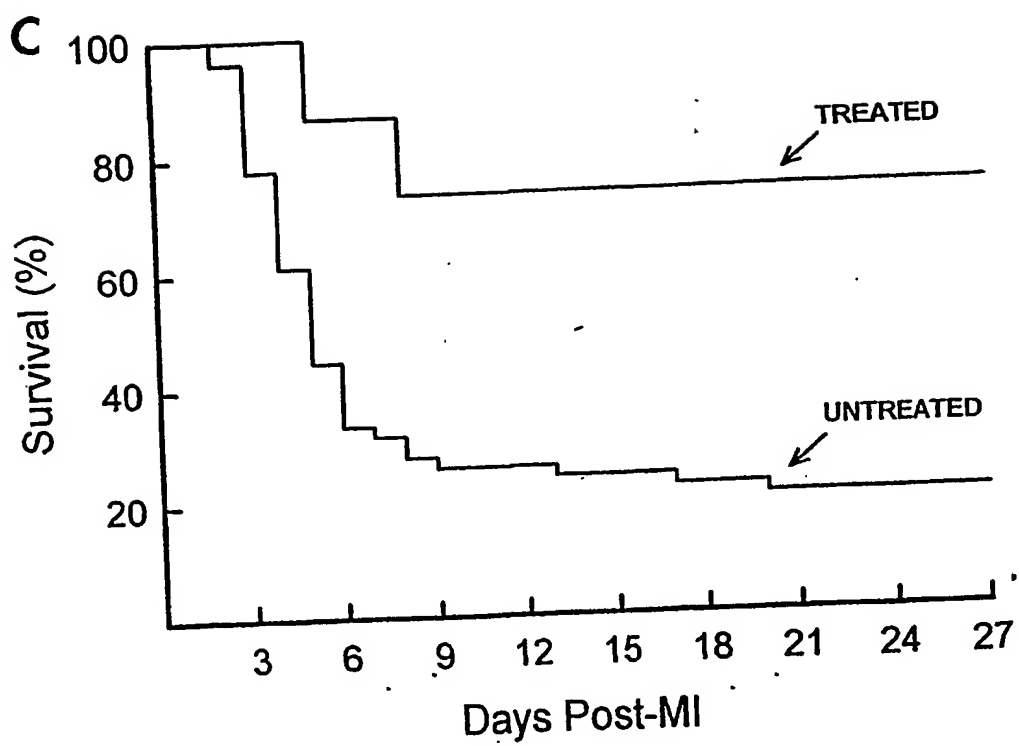


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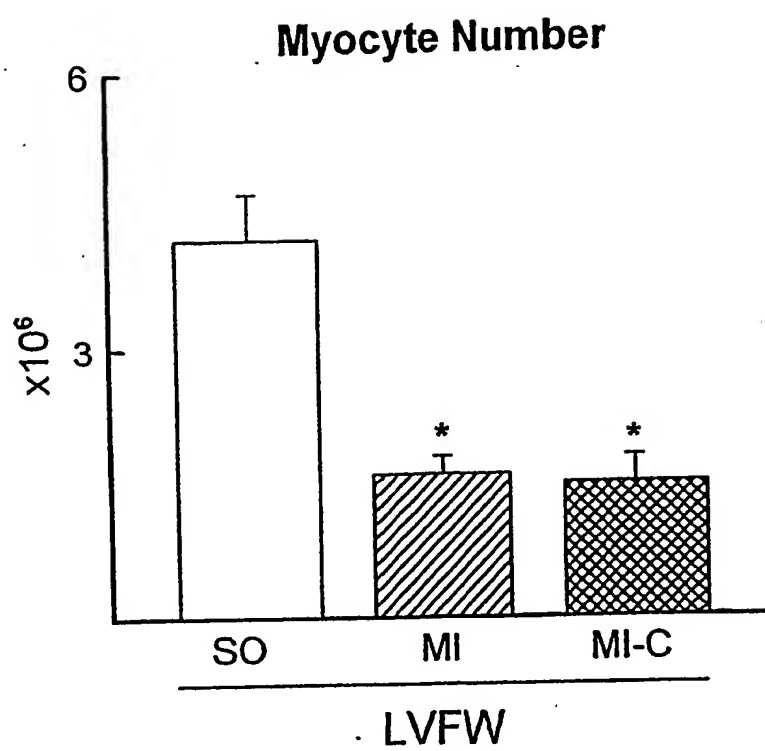


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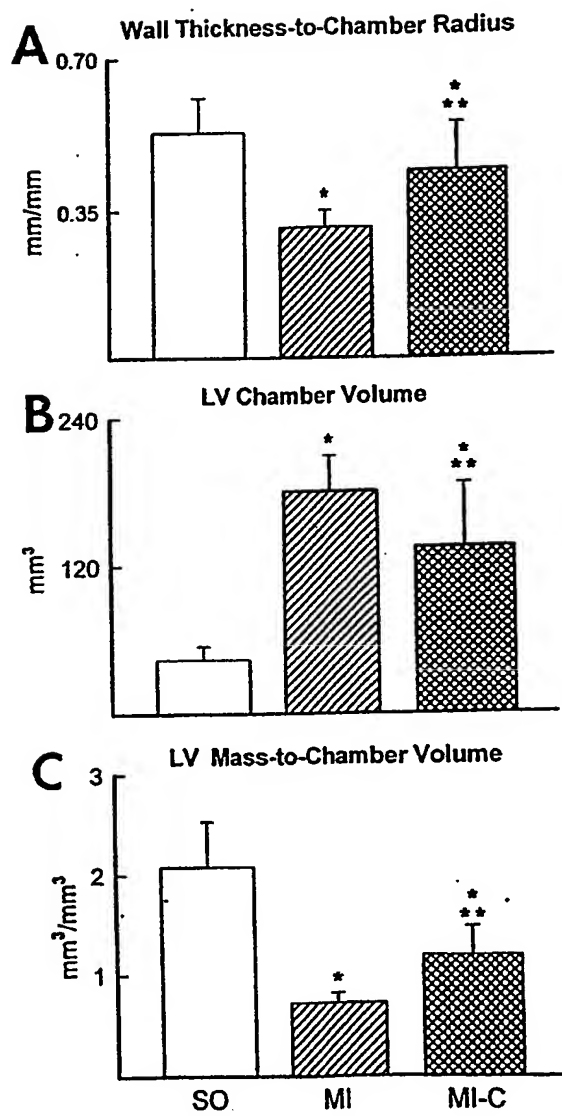


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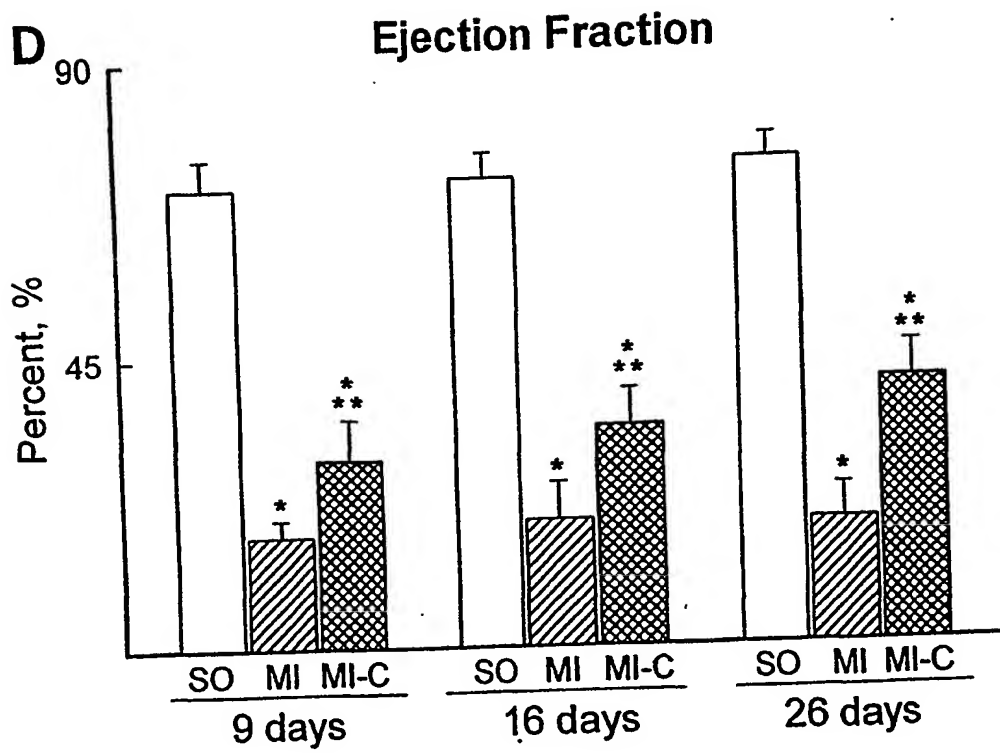


Figure 15

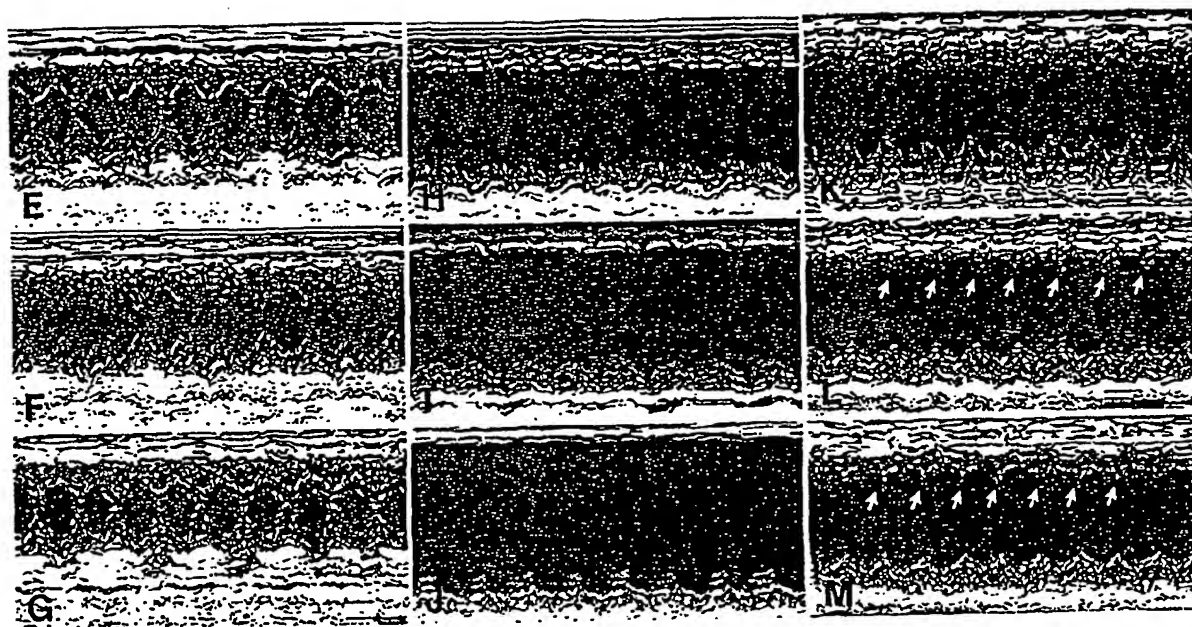


Figure 15

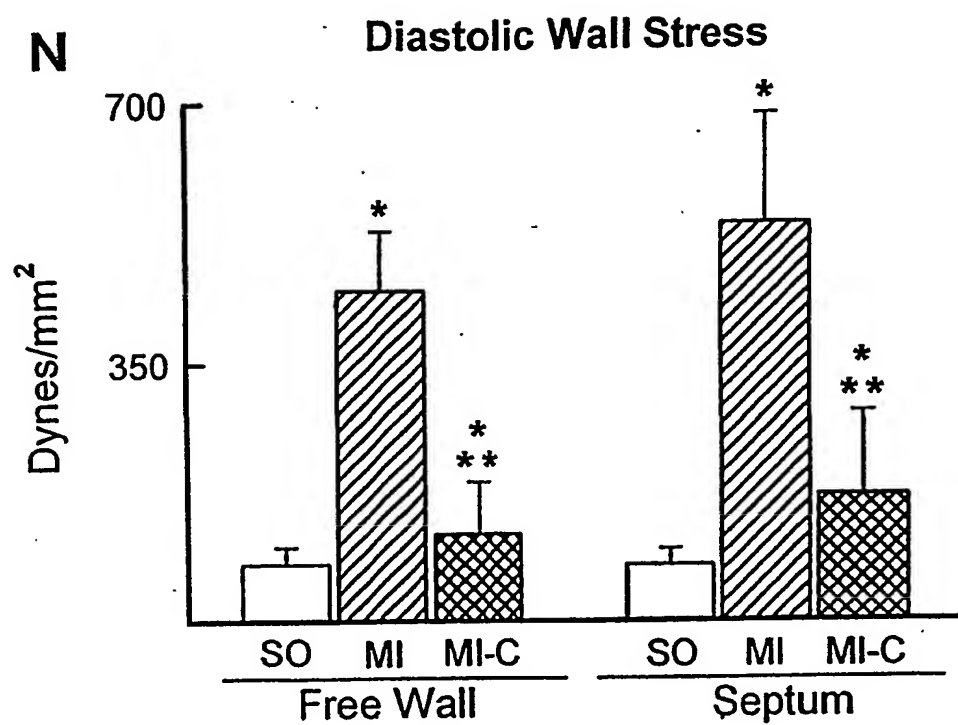


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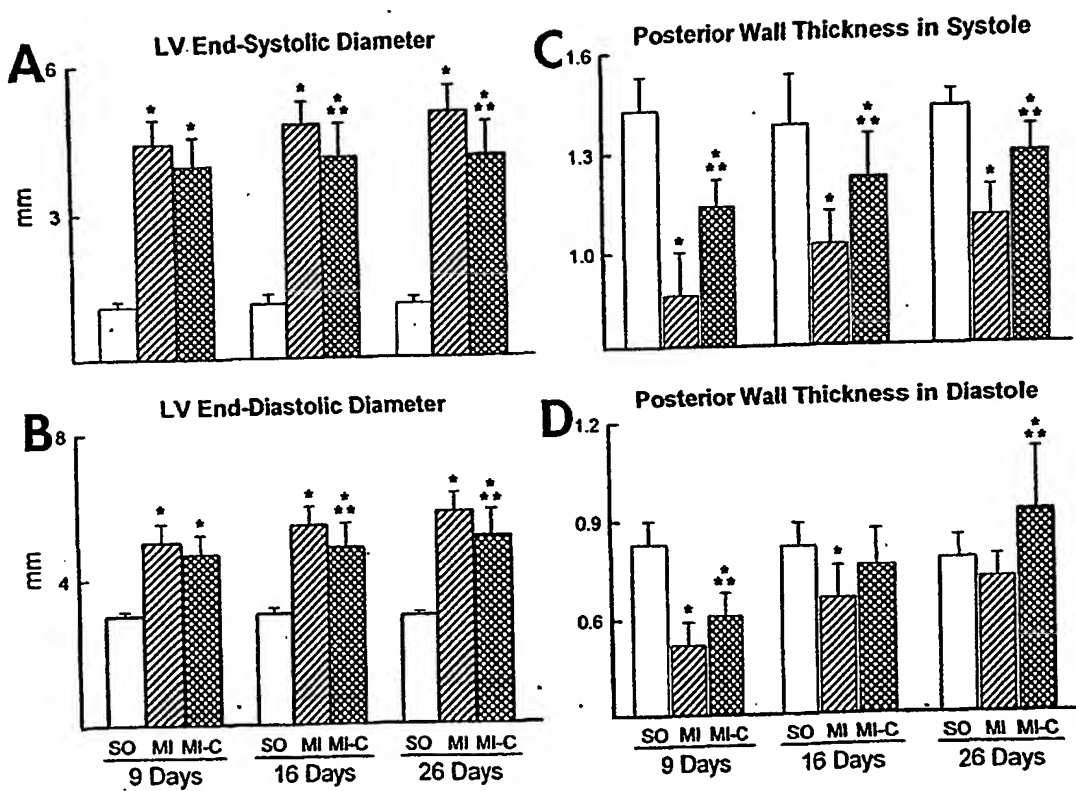


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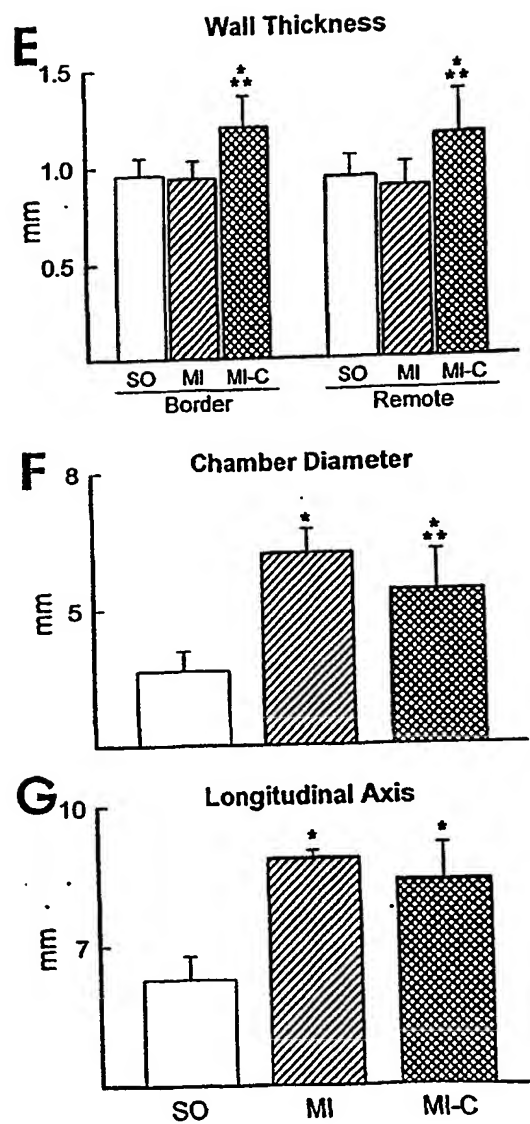


Figure 16

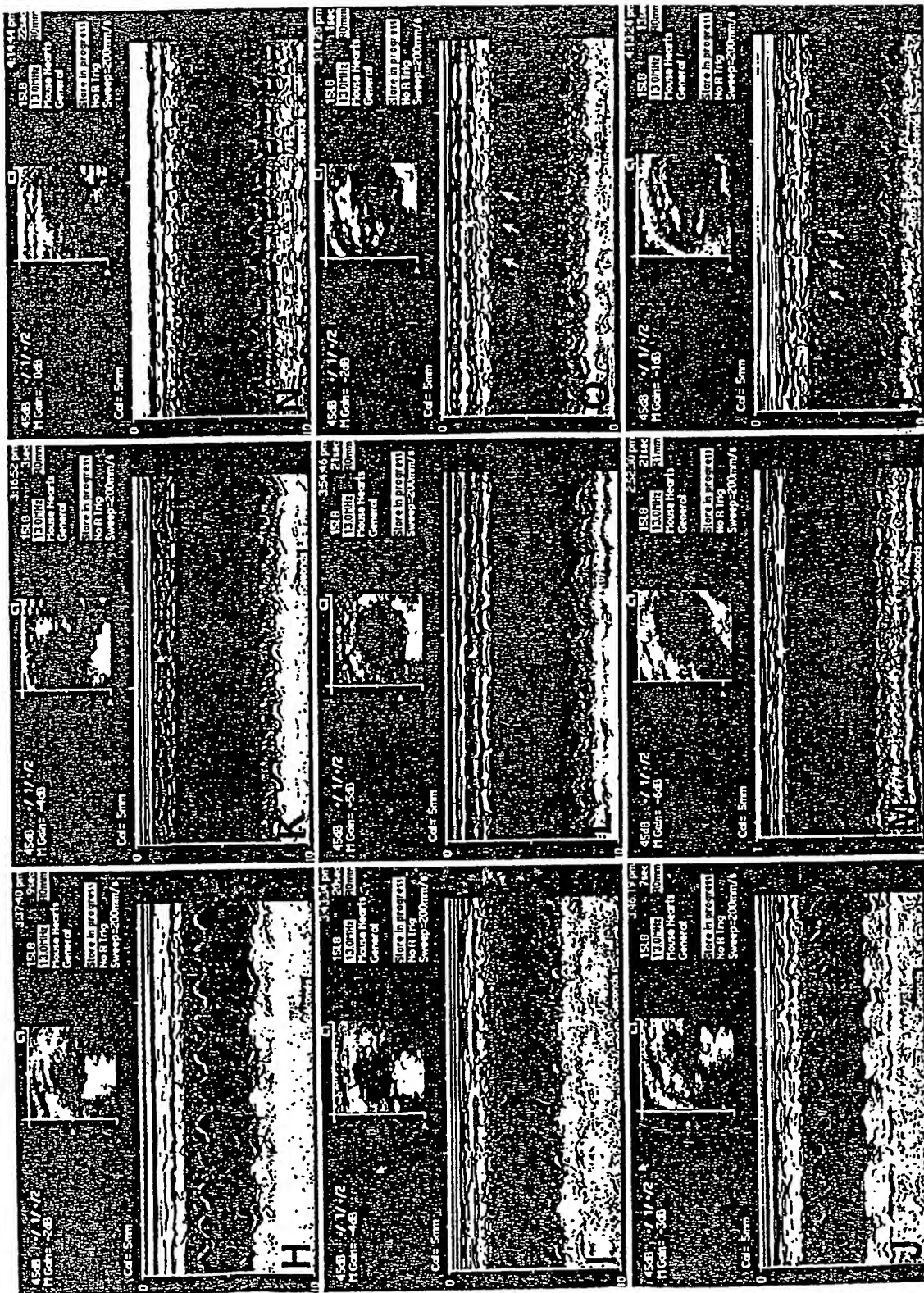


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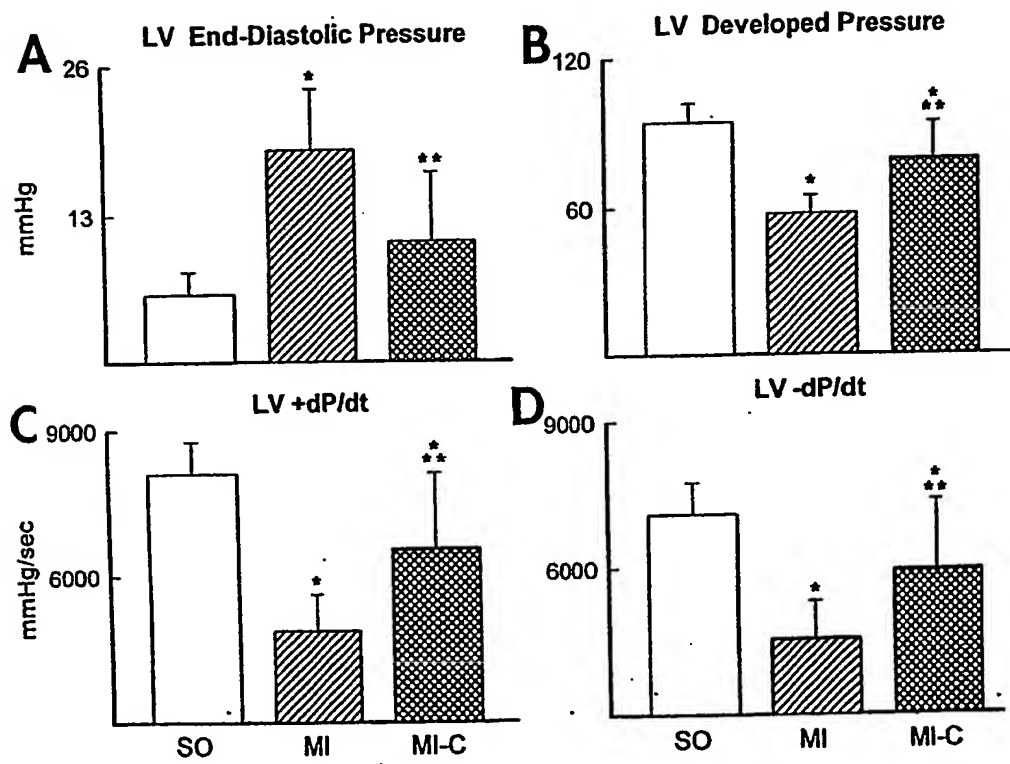


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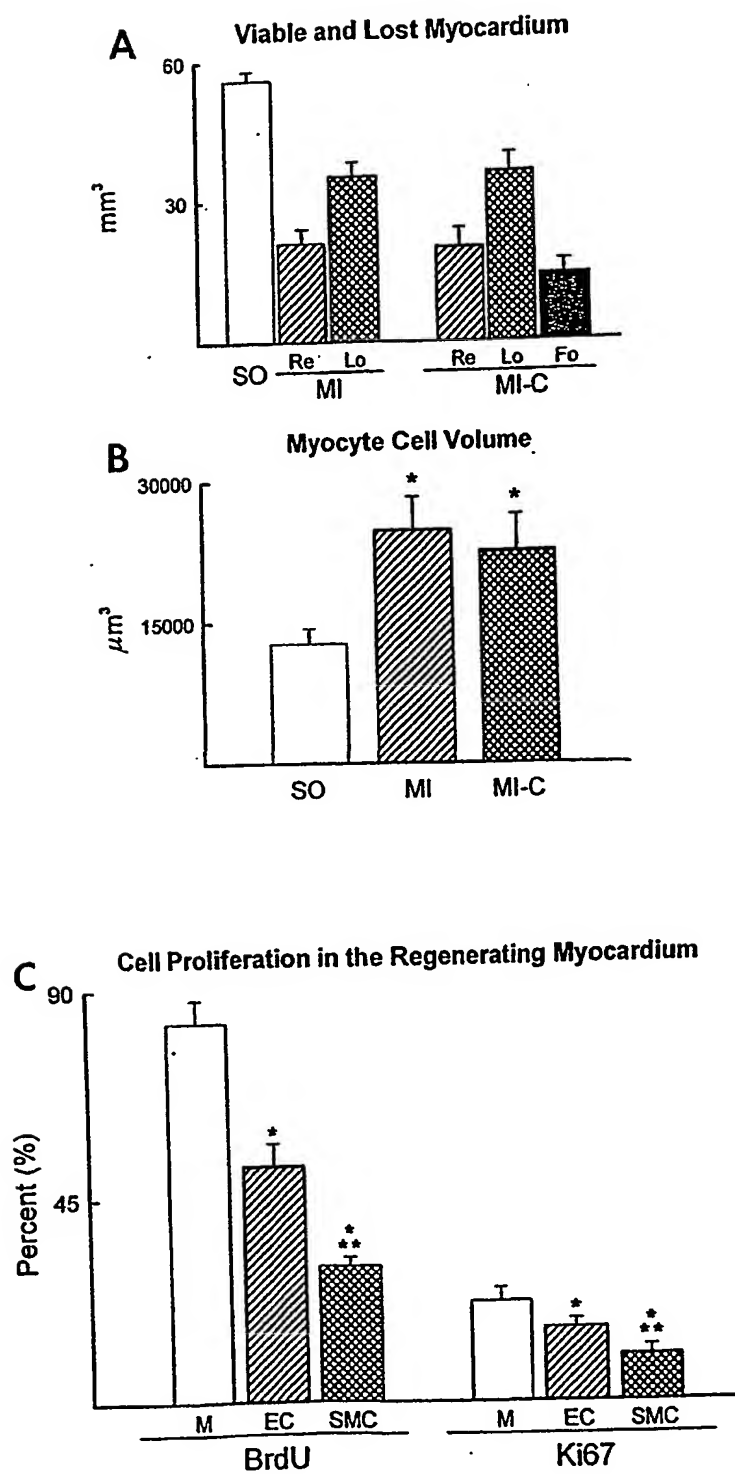


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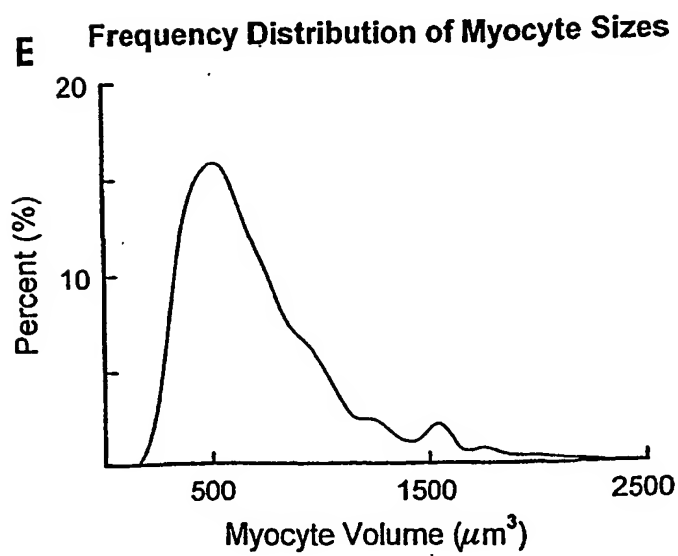
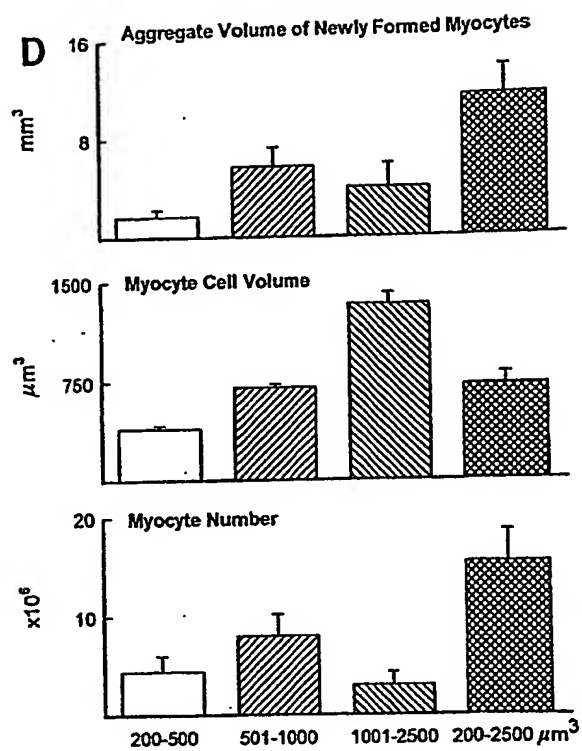


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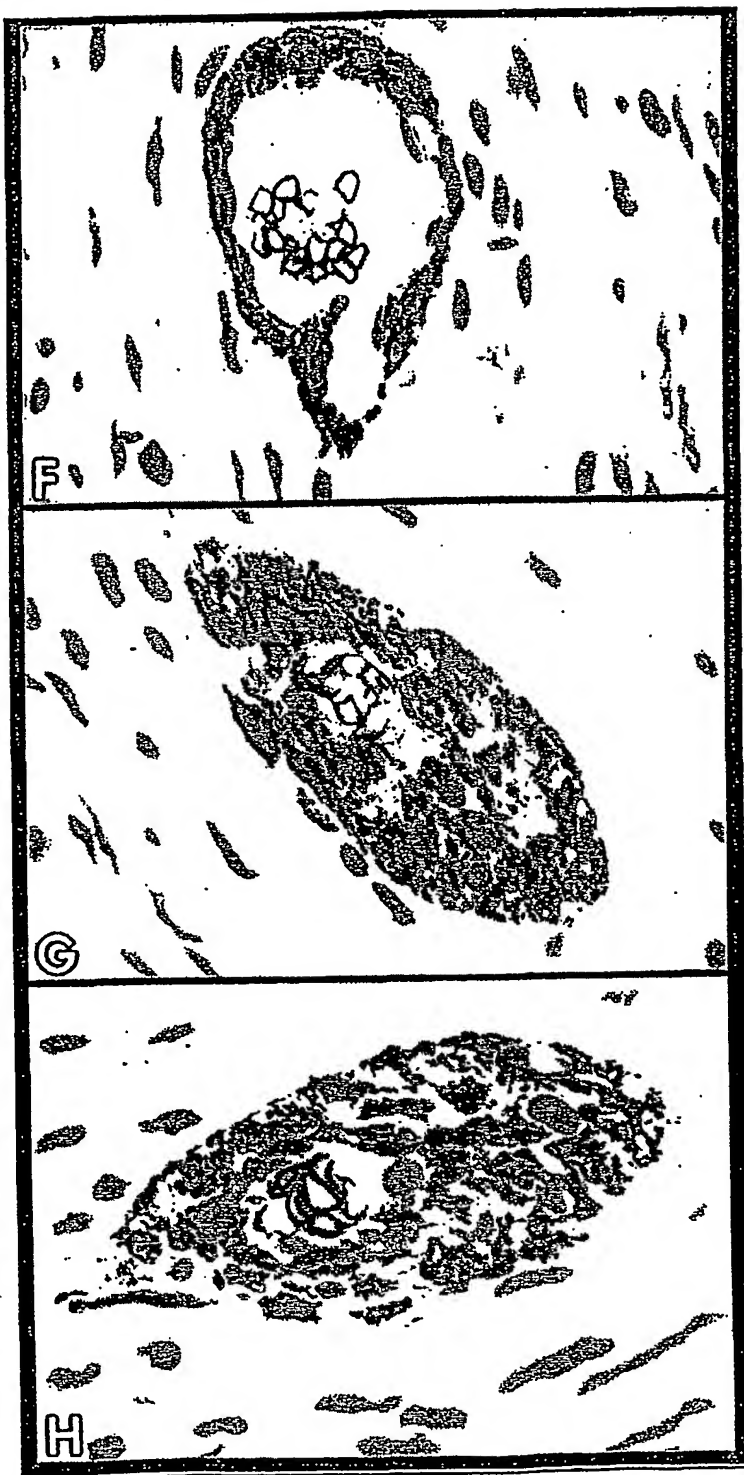


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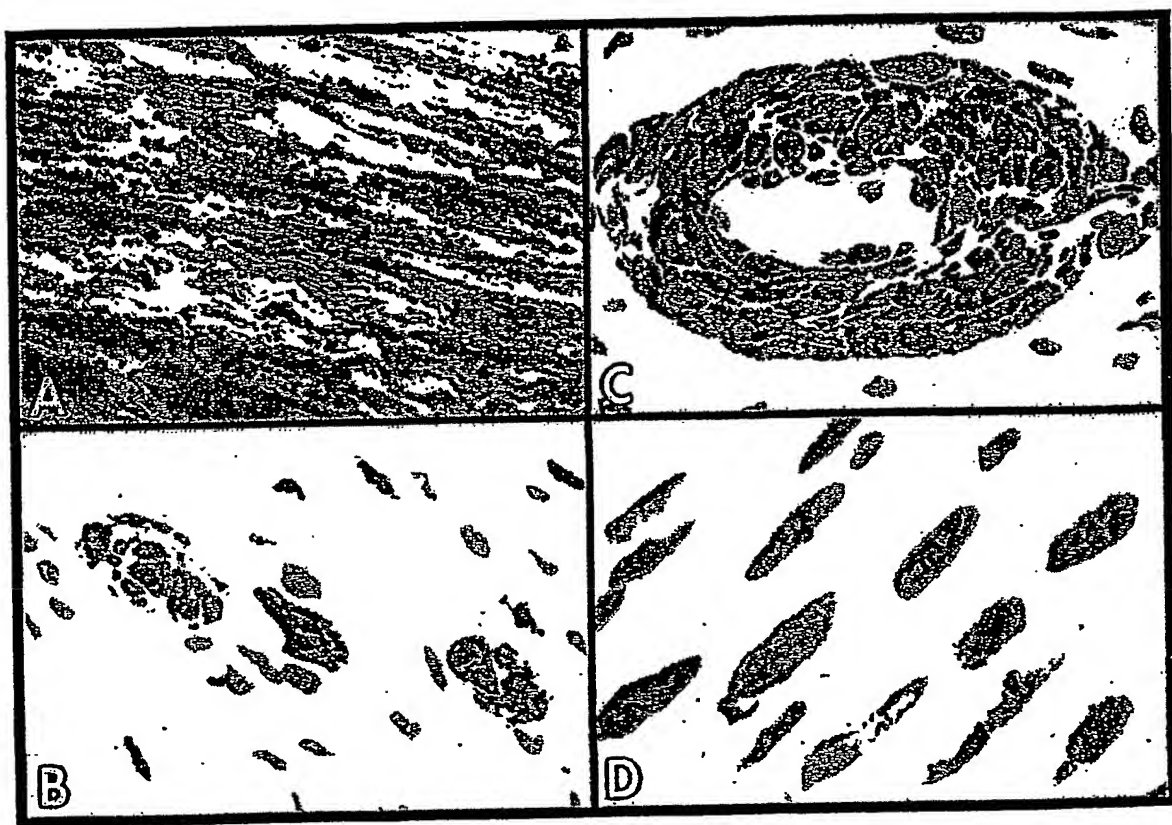


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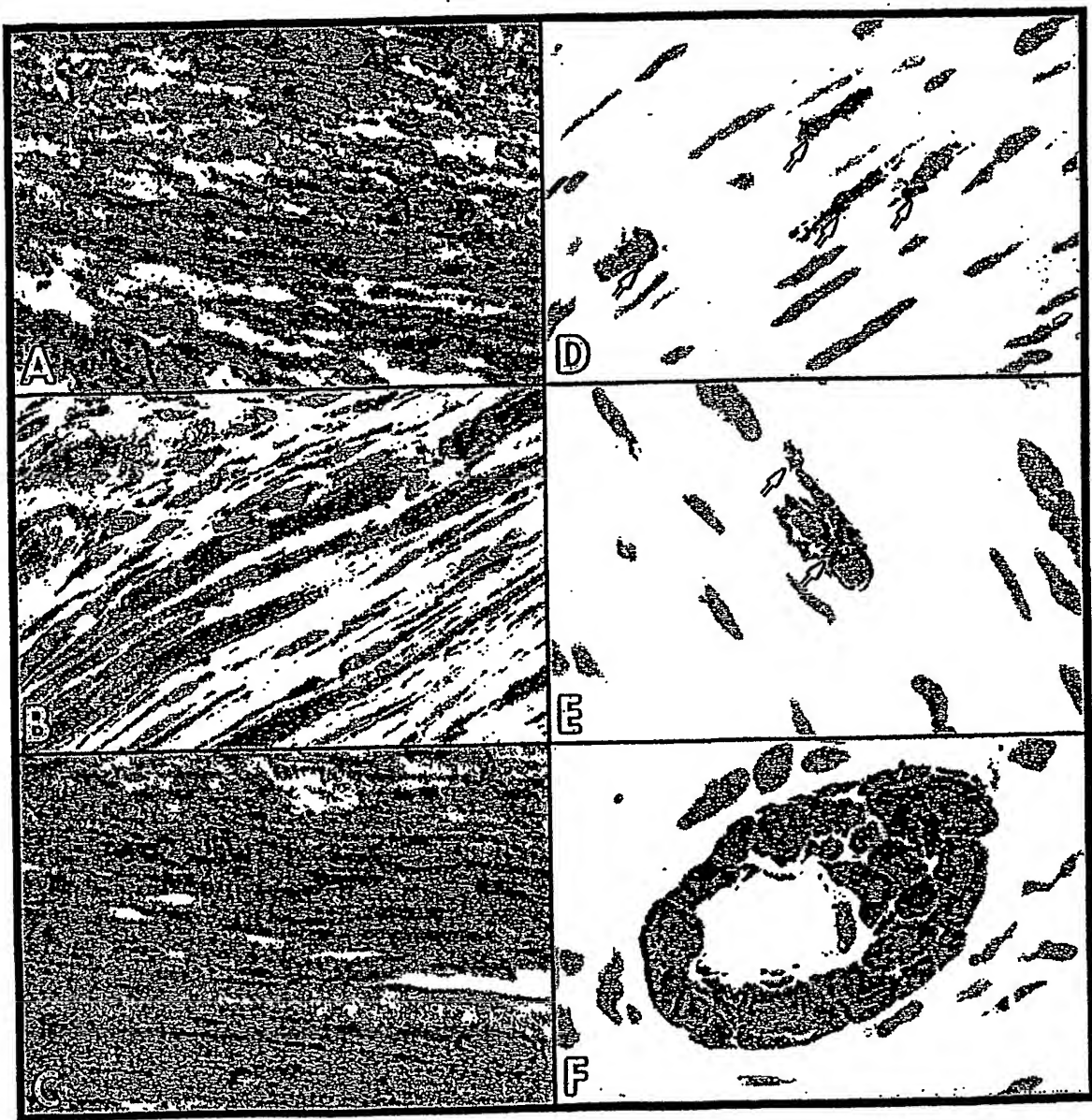


Figure 20

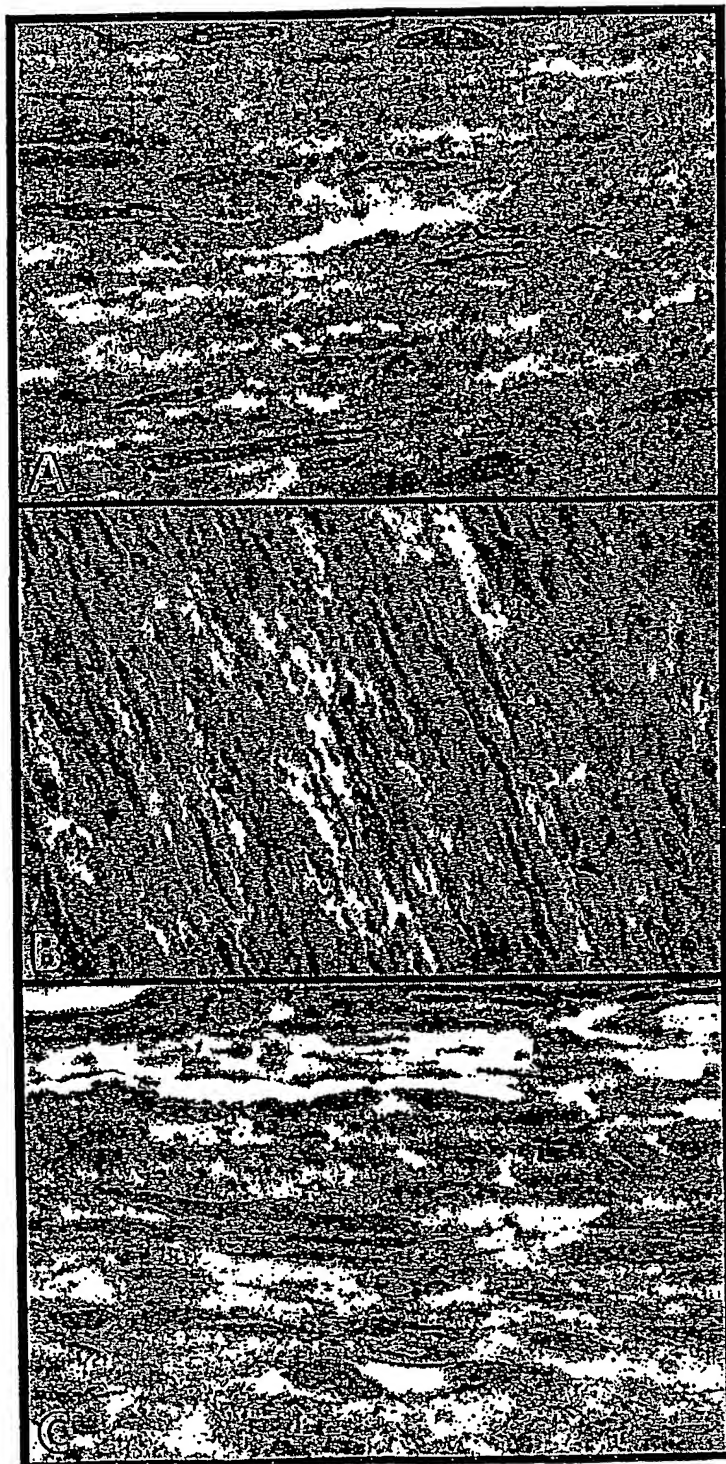


Figure 21